ADAPTACIONES FISIOLÓGICAS EN EL PAPAMOSCAS CERROJILLO (*FICEDULA HYPOLEUCA*): ESTRÉS OXIDATIVO, REPRODUCCIÓN Y DESARROLLO

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PHYSIOLOGICAL ADAPTATIONS IN THE PIED FLYCATCHER (*FICEDULA HYPOLEUCA*): OXIDATIVE STRESS, REPRODUCTION AND DEVELOPMENT

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RESUMEN

El estrés oxidativo es el desequilibrio entre la capacidad antioxidante de un organismo y la producción de radicales libres que pueden dañar importantes biomoléculas (lípidos, proteínas o ADN), afectando al buen funcionamiento celular. Las condiciones ambientales experimentadas por los organismos pueden ejercer una fuerte influencia sobre el desarrollo y expresión de su fenotipo, especialmente durante el desarrollo temprano y la reproducción, lo que puede determinar el acceso a territorios o incluso la fecundidad y la supervivencia. Por otro lado, entender la relación que existe entre el estado oxidativo y el acortamiento de los telómeros -regiones de ADN no codificante cuya función es estabilizar la estructura de los cromosomas y con un papel fundamental en el envejecimiento-, puede desentrañar los mecanismos que subyacen a las estrategias vitales determinadas por los compromisos existentes entre reproducción, mantenimiento y crecimiento. Así, el objetivo general de esta tesis es determinar, desde un punto de vista ecológico-evolutivo, el papel del estrés oxidativo en relación a las distintas estrategias vitales desarrolladas por las aves, analizando la influencia del ambiente de nidificación y los factores intrínsecos y externos que afectan a los individuos durante el desarrollo temprano y la reproducción.

En general, se han estudiado las adaptaciones fisiológicas, en relación al estrés oxidativo, a los diferentes compromisos a los que se enfrentan las aves a lo largo de su ciclo vital. Se ha utilizado el Papamoscas cerrojillo (*Ficedula hypoleuca*) como modelo de estudio. Las zonas de estudio están enclavadas en la Sierra de Guadarrama, una situada en los Montes de Valsaín, Segovia, donde hay colocadas 570 cajas-nido y otra en Lozoya, Madrid, con 100 cajas-nido. Para la realización de esta tesis, se han llevado a cabo tanto estudios experimentales como observacionales para analizar la influencia del ambiente de nidificación y los parásitos del nido sobre polluelos e individuos parentales, los costes derivados de la inversión reproductiva y el mantenimiento de ornamentos sexuales, los factores que afectan al estado redox en la edad temprana y las relaciones entre reproducción, estrés oxidativo y envejecimiento. Se han realizado filmaciones de los nidos para analizar el cuidado parental y en todos los casos se han tomado muestras de sangre a todos los individuos, adultos y pollos, para análisis de parámetros oxidativos y bioquímicos (daño oxidativo, antioxidantes, triglicéridos, ácido úrico), sexaje (sólo en pollos) y medidas de telómeros (adultos). Además se han tomado fotografías de los ornamentos del plumaje de adultos para analizarlos como señal de calidad individual.

Esta tesis contribuye a comprobar la fuerte implicación del estado oxidativo en los compromisos que sustentan a las estrategias vitales de las aves. Por un lado en esta tesis se muestra que la presencia de material viejo en el nido no afecta a todas las poblaciones de ectoparásitos por igual, cuestionando la idea general de que la reutilización de nidos está ligada a mayores infestaciones, con consecuencias sobre el éxito reproductor y el crecimiento de polluelos. Además, se muestra cómo el método de manipulación de la carga de ectoparásitos del nido puede tener efectos no controlados sobre las aves, lo que puede llevar a subestimar las consecuencias potenciales de la presencia de dichos ectoparásitos sobre sus hospedadores aviares. Se evidencian experimentalmente efectos negativos de la carga de ectoparásitos sobre el estado oxidativo de hembras adultas y polluelos en desarrollo, lo que podría acarrear consecuencias sobre la supervivencia y reproducción futuras. También se muestran las asociaciones entre los diferentes componentes del estado oxidativo de polluelos en desarrollo y varios factores ambientales e intrínsecos, algo que resulta esencial para entender la importancia del estrés oxidativo en la formación del fenotipo. Por otro lado se

sugiere que diferentes rasgos acromáticos del plumaje pueden señalizar la capacidad individual para hacer frente al estrés oxidativo y se resalta la importancia de las distintas fases del ciclo reproductor para entender el papel del estrés oxidativo como coste y limitación en la reproducción. Por último, se evidencia cómo el estado oxidativo está involucrado en un acortamiento más rápido de los telómeros (consecuentemente con un mayor envejecimiento celular) de los individuos en edad adulta como coste de la reproducción, además de sugerir que la edad fisiológica de los padres puede afectar a la calidad de la descendencia en términos de estrés oxidativo. Este entramado de relaciones demuestra que la ecología de los organismos es en parte ecología oxidativa. En definitiva, esta tesis abre nuevas vías para futuros estudios sobre estrategias vitales, comportamiento y ecofisiología en poblaciones naturales, sugiriendo que estos se beneficiarán de incluir entre los parámetros estudiados aquellos relacionados con el estado oxidativo de los individuos.

ABSTRACT

Oxidative stress is the imbalance between the antioxidant capacity of an organism and the production of free radicals that can damage important biomolecules (lipids, proteins or DNA), affecting cellular senescence. Environmental conditions experienced by organisms can exert a strong influence on the development and expression of their phenotype, especially during early development and reproduction, which may determine access to territories, fecundity and survival. On the other hand, understanding the relationship between oxidative status and telomere dynamics -regions of non-coding DNA whose function is to stabilize the structure of chromosomes and with a fundamental role in aging- is essential to fully appraise the underlying mechanisms to the life-histories determined by the existing trade-offs between reproduction, maintenance and growth. Thus, the general objective of this thesis is to determine, from an ecological-evolutionary point of view, the role of oxidative stress in relation to the different life-histories developed by the birds, analyzing the influence of the nesting environment and the intrinsic

and external factors affecting individuals during early development and reproduction.

In general, we have studied the physiological adaptations, in relation to oxidative stress, to the different trade-offs that birds face during their life cycle. The Pied flycatcher (*Ficedula hypoleuca*) has been used as the study model. The study areas are located in the Sierra de Guadarrama, one in the Montes de Valsaín, Segovia, where there are 570 nest boxes, and another one in Lozoya, Madrid, with 100 nest boxes. Both experimental and observational studies have been carried out to analyze the influence of the nesting environment and the nest-dwelling ectoparasites on nestlings and parental individuals, the costs derived from the reproductive investment and the maintenance of sexual ornaments, the factors affecting the redox status in the early life and the relationships between reproduction, oxidative stress and ageing. Films of nests have been made to analyze parental care and, in all cases, blood samples have been taken from all individuals, adults and nestlings, for analysis of oxidative and biochemical parameters (oxidative damage, antioxidants, triglycerides, uric acid), sex (only in nestlings) and measures of telomeres (adults). In addition, photographs of adult plumage ornaments have been taken to analyze them as a sign of individual quality.

This thesis contributes to verify the strong implication of the oxidative state in the trade-offs that sustain life-histories of birds. On the one hand, this thesis shows that the presence of old material in the nest does not affect all populations of ectoparasites equally, questioning the general idea that nest reuse is linked to higher infestations, with consequences on reproductive success and nestling growth. In addition, it is shown how the method to reduce ectoparasite loads of the nest can have uncontrolled effects on birds, which may lead to underestimation of the potential consequences of the presence of such ectoparasites on their avian hosts. Negative effects of ectoparasite loads on the oxidative status of adult females and developing chicks are evidenced experimentally, which could have consequences on future survival and reproduction. It also shows the associations between different components of the oxidative status of developing chicks and various environmental and intrinsic factors, which is essential to understand the importance of oxidative stress in the formation of the phenotype. On the other hand, it is suggested that different achromatic features of plumage can signal the

individual capacity to cope with oxidative stress and the importance of the different phases of the reproductive cycle is emphasized to understand the role of oxidative stress as cost and constraint in reproduction. Finally, it is evidenced how the oxidative status is related with telomeres shortening (consequently with a greater cellular ageing) of the individuals in adulthood like cost of the reproduction, in addition to suggesting that the physiological age of the parents can affect the quality of the offspring in terms of oxidative stress. This framework of relationships shows that the ecology of organisms is partly oxidative ecology. In short, this thesis opens new avenues for future studies on life-histories, behaviour and ecophysiology in natural populations, suggesting that these will benefit from including different oxidative parameters of individuals.

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Memoria presentada por la Licenciada Jimena López Arrabé para optar al grado de Doctor por la Universidad Autónoma de Madrid, dirigida por el Dr. Juan Moreno Klemming del Museo Nacional de Ciencias Naturales-CSIC y el Dr. Lorenzo Pérez Rodríguez de la Estación Biológica de Doñana-CSIC.

Madrid, 2017

El Doctorando

Jimena López Arrabé

Vº Bº del Director

Juan Moreno Klemming

Vº Bº del Director

Lorenzo Pérez Rodríguez

A mis padres y a mi hermana

A Fer y a Martín

There are some four million different kinds of animals and plants in the world. Four million different solutions to the problems of staying alive.

Sir David Attenborough

Life on Earth, 1979

ÍNDICE

La integración de la ecología y la fisiología es clave para entender los mecanismos que subyacen a la evolución de las diferentes estrategias vitales y a la forma en la que los organismos responden a distintas presiones ambientales. La teoría de las historias de vida ("*life-history theory*") proporciona un marco conceptual para el estudio de dichas estrategias vitales que engloban rasgos asociados al éxito reproductor y la supervivencia de los organismos. Uno de los principios fundamentales es que la evolución de estos rasgos viene limitada por la existencia de compromisos entre ellos (Stearns, 1992). Estos compromisos se han asociado habitualmente a la distribución de recursos o energía entre crecimiento, reproducción o supervivencia (Dowling & Simmons, 2009; Monaghan *et al.*, 2009), de modo que la inversión de un recurso limitado en un carácter tiene consecuencias negativas sobre otros caracteres que también necesiten de ése recurso (Zera & Harshman, 2001).

Sin embargo, los compromisos también podrían producirse como resultado de actividades que generan consecuencias negativas sobre caracteres no directamente implicados en el compromiso (Dowling & Simmons, 2009; Monaghan *et al.*, 2009). En este sentido, el estrés oxidativo estudiado en el contexto de la ecología evolutiva ha sido señalado como unos de los mecanismos comunes a muchos procesos biológicos de los animales e implicado, por tanto, en la evolución de las diferentes estrategias vitales observadas en la naturaleza.

1. EL ESTRÉS OXIDATIVO

La transición de una química reductora a una química oxidante en la atmósfera terrestre hace alrededor de unos 2.4 billones de años en lo que se conoce como la Gran Oxidación (Sessions *et al.*, 2009), desencadenó el camino para la diversificación de la vida, expandiendo las capacidades metabólicas y bioquímicas

de los organismos e incrementando su complejidad (Costantini, 2014). Sin embargo, el uso del oxígeno como recurso implicó la evolución de distintos mecanismos para mitigar los efectos tóxicos generados por radicales libres y otras especies reactivas derivadas del proceso de obtención de energía en la cadena respiratoria. De este modo, la relación entre la generación de energía y el daño oxidativo está modulada por el desarrollo de sofisticados sistemas antioxidantes (Monaghan *et al.*, 2009).

El estrés oxidativo es el desequilibrio entre la producción de compuestos pro-oxidantes (especies reactivas de oxígeno y nitrógeno, ERONs) y la maquinaria de defensas antioxidantes de la que dispone el organismo (Halliwell & Gutteridge, 2007). Cuando los niveles de producción de estas moléculas pro-oxidantes superan la capacidad antioxidante del individuo, pueden quedar libres y oxidar componentes celulares, provocando daños oxidativos a importantes biomoléculas (lípidos, proteínas o ADN) y afectando a su funcionalidad en el organismo (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2007).

Esta situación de desequilibrio se puede dar por dos motivos no excluyentes: debido a un aumento en la producción de especies reactivas o de moléculas reactivas secundarias derivadas de la oxidación, o bien debido a una disminución en los niveles de defensas antioxidantes o en los sistemas de reparación (Figura 1).

1.1. Pro-oxidantes

Las moléculas pro-oxidantes son aquellas especies bioquímicas reactivas, altamente inestables, capaces de generar oxidación. Pueden ser radicales libres y no radicales. Las moléculas pro-oxidantes más importantes son las ERONs, cuyos electrones desemparejados captan electrones de otros átomos, produciendo nuevas especies reactivas y dando lugar a un proceso en cascada (Monaghan *et al.*, 2009). Aunque estas moléculas pro-oxidantes pueden tener un origen exógeno (ej. tóxicos), el principal lugar de generación es la mitocondria, durante la respiración celular (Dowling & Simmons, 2009).

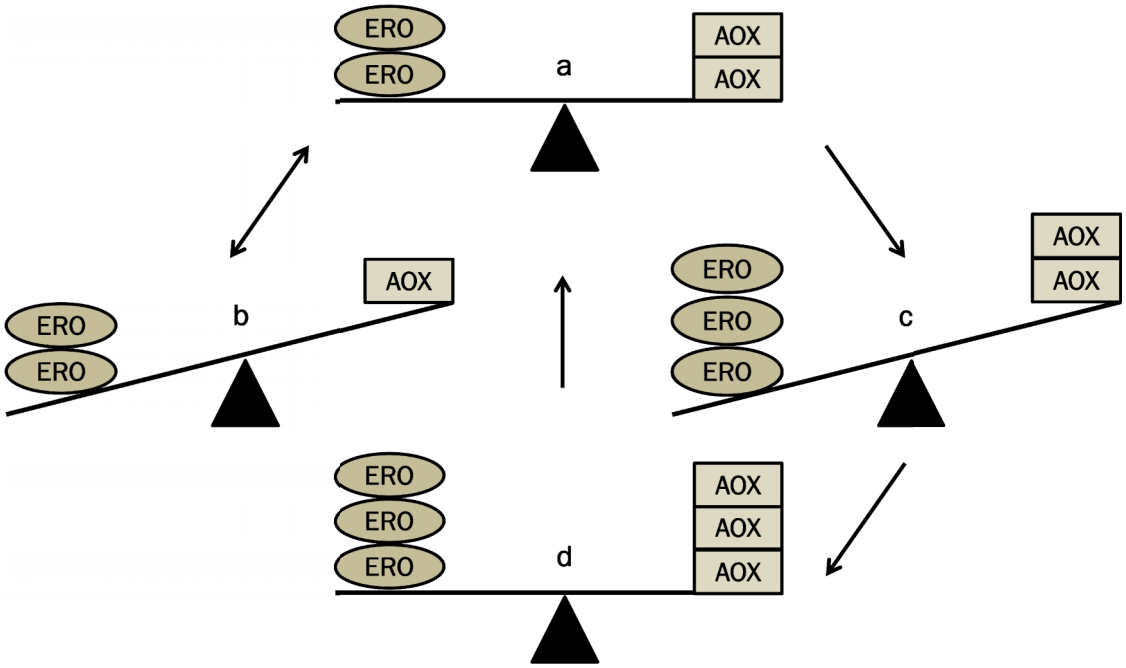

Figura 1. *Representación gráfica de de las relaciones existentes entre las especies reactivas del oxígeno (ERO) y los sistema de defensa antioxidante (AOX).* ***a)*** *en condiciones normales de homeostasis basal, los niveles de ERO y AOX son bajos y están equilibrados. No hay estrés oxidativo;* ***b)*** *situación de estrés oxidativo por una disminución en los niveles de AOX. Esta situación se puede revertir si vuelven a aumentar los AOX;* ***c)*** *situación de estrés oxidativo por aumento de los niveles de ERO, sobrepasando a los AOX;* ***d)*** *aumento de los AOX para hacer frente al incremento de ERO. Si la compensación es exitosa, se vuelve a la homeostasis basal de nuevo. (Modificado de Monaghan* et al.*, 2009).*

1.2. Daño oxidativo

El principal efecto negativo que producen estas especies reactivas es el daño oxidativo en importantes biomóleculas como son el ADN, las proteínas o los lípidos del organismo.

La oxidación de bases puede tener consecuencias serias a nivel fenotípico, pudiendo generar mutaciones y carcinogénesis. El ADN mitocondrial es el más vulnerable a esta oxidación por estar más expuesto (Finkel & Holbrook, 2000; Monaghan *et al.*, 2009). Los telómeros, los extremos de los cromosomas compuestos por ADN no codificante que da estabilidad al genoma, también son vulnerables al ataque de especies reactivas, y una reducción acelerada en su longitud derivada del estrés oxidativo puede dar lugar a una rápida senescencia celular (Von Zglinicki, 2002).

En el caso de las proteínas, la oxidación de aminoácidos puede alterar su estructura secundaria y terciaria, provocando una pérdida de funcionalidad (Surai, 2002).

Pero es en los lípidos donde se producen los mayores daños, por ser las moléculas más expuestas al estrés oxidativo. La peroxidación lipídica es la oxidación de lípidos durante la cual se producen radicales libres y peróxidos a partir de los ácidos grasos insaturados con efectos citotóxicos y consecuencias graves para la función y estructura de las membranas celulares (Hulbert *et al.*, 2007). Además, el daño oxidativo a lípidos puede tener efectos más amplios, ya que provoca una reacción en cadena en la que se ven involucrados productos intermedios y finales de la peroxidación lipídica, como el malondialdehído (MDA), que también pueden producir alteraciones en las proteínas y el ADN (Hulbert *et al.*, 2007; Monaghan *et al.*, 2009).

1.3. Antioxidantes

Para hacer frente a los efectos negativos de las especies reactivas, los organismos disponen de sofisticados sistemas antioxidantes que actúan retrasando, reduciendo o eliminando el daño oxidativo.

Aquellos antioxidantes que actúan principalmente durante una situación de desequilibrio redox ("oxidación-reducción") se dividen en dos grandes grupos: los enzimáticos, como la superóxido dismutasa (SOD), la catalasa (CAT) o la glutatión peroxidasa (GPX), y los no enzimáticos, que incluyen moléculas de síntesis endógena (ej. ácido úrico, glutatión reducido, vitamina C) y antioxidantes que han de obtenerse a través de la dieta (ej. carotenoides, vitamina E) (Halliwell & Gutteridge, 2007).

Además de estos antioxidantes, existen otros mecanismos de defensa que actúan de una forma más indirecta, antes y después de que se produzca la situación de desequilibrio. Estos se agrupan en defensas estructurales (algunos tejidos importantes poseen una estructura más resistente debido a su composición de aminoácidos y ácidos grasos), mecanismos para minimizar la producción de

especies reactivas dentro de la célula y sistemas de reparación una vez que se ha producido el daño (Monaghan *et al.*, 2009).

1.4. Ecología evolutiva y estrés oxidativo

Desde un punto de vista ecológico-evolutivo, el estrés oxidativo suscita un gran interés debido a que es un coste potencial inherente a la vida animal y que puede suponer una limitación importante para muchos procesos biológicos (Costantini, 2008; Monaghan *et al.*, 2009). De este modo, el estrés oxidativo puede afectar a la capacidad de los animales para sobrevivir y reproducirse, y está detrás del diseño fenotípico de los organismos, lo que hace de él un tema de estudio con un gran potencial explicativo en ecología. Además, determinar los distintos factores que afectan al estado oxidativo de un individuo es fundamental para obtener una visión global de los compromisos que subyacen a las estrategias vitales.

Gran parte de los trabajos eco-fisiológicos sobre estrés oxidativo se centran en las aves como modelo de estudio. Las aves generalmente tienen una vida larga con menores tasas de envejecimiento en comparación con mamíferos del mismo tamaño. Sin embargo poseen tasas metabólicas y un gasto energético más elevados, lo que en teoría debería acelerar el daño celular y la senescencia fisiológica. Sin embargo, parece que las aves han desarrollado adaptaciones únicas para protegerse frente al estrés oxidativo, características que las hacen idóneas como modelo de estudio en este campo (Costantini, 2008).

En esta tesis se han estudiado diversas adaptaciones fisiológicas relacionadas con el estrés oxidativo a lo largo de distintas etapas del ciclo vital, especialmente durante el desarrollo y la reproducción, en el Papamoscas cerrojillo (*Ficedula hypoleuca*), una especie de ave paseriforme de pequeño tamaño que nidifica en oquedades.

2. AMBIENTE DE NIDIFICACIÓN Y ESTADO OXIDATIVO

Los nidos de las aves son estructuras construidas por los adultos durante el período reproductor para albergar la puesta y el posterior desarrollo de las crías. Son considerados como parte del fenotipo extendido de las aves (Dawkins, 1982), y están diseñados para proteger a la nidada mediante la proporción de un ambiente controlado que favorecerá la supervivencia (Hansell, 2000). En el caso de aquellos nidos construidos dentro de cavidades, éstas ofrecen ambientes microclimáticos más estables y un menor riesgo de depredación en comparación con los nidos abiertos (Collias & Collias, 1984; Nilsson, 1984; Hansell, 2000). Sin embargo, una mayor estabilidad de temperatura y humedad dentro de la cavidad ofrece unas condiciones excelentes para la proliferación de parásitos que habitan en los nidos y que se alimentan de la piel, plumas y sangre de sus hospedadores (Collias & Collias, 1984).

La reutilización de nidos por aves que habitan en cavidades no es muy común, sin embargo, en ocasiones una baja disponibilidad de oquedades apropiadas para la cría puede hacer que algunas parejas ocupen agujeros que contienen nidos viejos, reutilizando los mismos o aportando nuevo material encima del que ya había (Loye & Carroll, 1998; Mazgajski, 2007; Tomás *et al.*, 2007). Aunque esto puede tener algunos beneficios para las nuevas parejas al reducir los costes asociados a la construcción de nuevos nidos (Møller, 1990; Mappes *et al.*, 1994; Moreno *et al.*, 2010), la presencia de material viejo en el nido puede contener y/o atraer a más ectoparásitos que el material fresco (Mazgajski, 2007).

Los ectoparásitos del nido que se alimentan de la sangre de polluelos y adultos constituyen una importante fuerza selectiva que afecta a la evolución de las estrategias vitales (Møller, 1993). La presencia de ectoparásitos hematófagos en el nido (Figura 2) puede resultar en costes para los polluelos al comprometer su crecimiento y condición física (Richner *et al.*, 1993; Heeb *et al.*, 1998, 2000; Tomás *et al.*, 2008; Brommer *et al.*, 2011; Cantarero *et al.*, 2013) debido a una pérdida directa de nutrientes y a una reducción de la capacidad metabólica derivadas de su actividad alimentaria (Simon *et al.*, 2004). Asimismo puede afectar a los adultos, no solo por el efecto directo de su actividad hematófaga sobre ellos (por ejemplo

durante la incubación), sino también intensificando los comportamientos de higiene del nido para reducir la carga de ectoparásitos e incrementando el esfuerzo parental para tratar de compensar los efectos negativos sobre su descendencia (Oppliger *et al.*, 1994; Richner & Tripet, 1999; Cantarero *et al.*, 2013).

Figura 2. *Ectoparásitos hematófagos habitualmente presentes en los nidos de Papamoscas cerrojillo en las zonas de estudio. Arriba a la derecha ácaro de la especie* Dermannysus gallinoides*. Arriba a la izquierda pulga adulta de la especie* Ceratophyllus gallinae*. Abajo larva de mosca de la especie* Protocalliphora azurea *enganchada a la pata de un polluelo de Papamoscas cerrojillo (Foto: A. Cantarero).*

Además, la activación de diferentes sistemas de defensa frente a parásitos, como la activación del sistema inmune o la respuesta inflamatoria producida durante una picadura y un aumento de la actividad metabólica, generan especies reactivas favoreciendo el desequilibrio redox y afectando al estado oxidativo del individuo (Møller *et al.*, 1994; Demas *et al.*, 1997; Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2007; Sorci & Faivre, 2009). De este modo, el estrés oxidativo puede considerarse como un coste inmediato del parasitismo y su estudio es clave para interpretar los compromisos entre crecimiento, reproducción y

mantenimiento de los individuos hospedadores (Costantini, 2008; Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010; Metcalfe & Monaghan, 2013).

El estudio de los efectos que los ectoparásitos tienen sobre la condición y la fisiología de las aves puede abordarse mediante la manipulación de la carga de éstos en el nido. Durante dicha manipulación es importante elegir métodos que minimicen los efectos sobre otras variables ambientales que puedan subestimar el impacto de los ectoparásitos sobre el hospedador. Asimismo, hay que controlar posibles efectos indeseados de la manipulación sobre las especies de estudio con el fin de evitar resultados erróneos derivados.

En este contexto, el uso de algunos insecticidas comerciales para reducir o eliminar la carga parasitaria en los nidos puede generar un ambiente tóxico para aquellos individuos que están en íntimo contacto con el material del nido. La presencia de xenobióticos en el nido puede alterar el estado de polluelos y adultos a nivel fisiológico, incluyendo alteraciones hematológicas y bioquímicas y afectando al sistema antioxidante de los individuos (Otitoju & Onwurah, 2005; Khan *et al.*, 2012). Estos efectos podrían comprometer el desarrollo y la futura supervivencia de aquellos individuos especialmente vulnerables como son los polluelos en crecimiento, cuyos sistemas de detoxificación son aún inmaduros.

3. EL ESTRÉS OXIDATIVO DURANTE EL DESARROLLO

Las condiciones experimentadas durante el desarrollo juvenil pueden afectar a la eficacia biológica del individuo. El fenotipo de los organismos es el resultado de la interacción entre genotipo y ambiente durante su desarrollo (West-Eberhard, 2003). Uno de los rasgos que se puede medir para cuantificar el desarrollo es el crecimiento, tomando como referencia medidas biométricas como el peso o la talla. Sin embargo, durante el desarrollo temprano, los niveles de estrés oxidativo pueden ser particularmente altos como resultado de las elevadas tasas metabólicas requeridas para el crecimiento, además de una cierta inmadurez en los sistemas antioxidantes de los individuos (Monaghan *et al.*, 2009; Metcalfe &

Alonso-Alvarez, 2010). Niveles altos de estrés oxidativo durante el crecimiento pueden tener consecuencias sobre la eficacia biológica a largo plazo (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2006, 2007a; revisión en Dowling & Simmons, 2009; Noguera *et al.*, 2011; revisión en Monaghan *et al.*, 2009).

En diferentes estudios con especies modelo se ha puesto de manifiesto la existencia de una contribución genética a los niveles de generación de metabolitos pro-oxidantes y a la capacidad de resistencia frente a la oxidación (Costantini & Dell´Omo, 2006; Kim *et al.*, 2010; Losdat *et al.*, 2014). La comparación entre individuos relacionados (es decir, padres e hijos) permite inferir la contribución relativa de los genes a la variabilidad de rasgos específicos (Falconer & Mackay, 1996), proporcionando una estimación de la heredabilidad (h^2) de dichos rasgos (Lynch & Walsh, 1998).

Sin embargo, las condiciones ambientales también afectan a los niveles de estrés oxidativo experimentados por los individuos en etapas tempranas de desarrollo (Costantini *et al.*, 2006; Rubolini *et al.,* 2006; Monaghan, 2008; Stier *et al.,* 2014). Entre los factores ambientales que pueden modular las interacciones entre fenotipo y genotipo en aves se encuentran el acceso a alimento, la presencia de parásitos o el grado de competencia en el nido, las condiciones climáticas o efectos parentales como la calidad del huevo (efectos maternos), (revisión en Metcalfe & Monaghan, 2001; Blount *et al.*, 2003; Alonso-Alvarez & Velando, 2012). El tamaño de nidada, por ejemplo, puede afectar a la cantidad de alimento recibido por los polluelos, a su tasa de crecimiento y a la cantidad de energía consumida por la competencia entre hermanos, con una sobreproducción derivada de radicales libres y la adquisición diferencial de antioxidantes, lo que puede influir en última instancia en el estado oxidativo de los polluelos (Alonso-Alvarez *et al.*, 2007a; Losdat *et al.*, 2010). A su vez, la cantidad y/o calidad del alimento que proporcionan los padres depende también de la productividad del medio ambiente, pudiendo variar estacionalmente (Martin, 1987). Así, la fecha de cría es también un factor crítico que puede determinar la condición de la descendencia, particularmente en especies que se enfrentan a restricciones ambientales opuestas: si crían demasiado pronto, las condiciones climáticas pueden ser aún adversas, mientras que si se retrasan demasiado, la disponibilidad de alimento

disminuye, por lo que en ambos caso, el porcentaje de éxito reproductor podría verse reducido (Brown & Brown, 2000).

4. ESTRÉS OXIDATIVO Y SEÑALES SEXUALES

La evolución de la coloración corporal, y en particular de los ornamentos sexuales, en los animales ha sido objeto de curiosidad y fascinación en el campo de la biología evolutiva (Costantini, 2014). La producción de señales sexuales secundarias es un componente clave en inversión reproductiva en aquellas especies con reproducción sexual (Darwin, 1871; Andersson, 1994).

La expresión de muchos de estos ornamentos está relacionada con la condición y la supervivencia de los organismos (Andersson, 1994; Von Schantz *et al.*, 1999), de modo que aquellos individuos más ornamentados estarán en mejor condición o presentarán una mayor calidad genética que aquellos con ornamentos menos exagerados. Para que estas relaciones se mantengan, en términos evolutivos, la expresión de estas señales sexuales ha de tener costes asociados a su producción y/o mantenimiento. Así, si son señales honestas, sólo los individuos de mayor calidad serán capaces de hacer frente a esos costes (Zahavi, 1975). Estos costes se han asociado tradicionalmente a factores limitantes como la disponibilidad de recursos, a limitaciones energéticas o al estado de inmunocompetencia (Folstad & Karter, 1992; Hill, 2000; Siefferman & Hill, 2007). Sin embargo, la expresión de ornamentos podría ser sensible al estado oxidativo de los individuos, siendo el estrés oxidativo un importante nexo entre la producción y mantenimiento de señales sexuales y la variación en caracteres individuales relacionados con la eficacia biológica (Von Schantz *et al.*, 1999; Alonso-Alvarez *et al.*, 2007b).

La mayor parte de los estudios acerca del papel del estrés oxidativo en la expresión de ornamentos del plumaje se ha centrado principalmente en coloraciones basadas en carotenoides y en señales melánicas (ej. Galván & Alonso-Alvarez, 2008; Pérez-Rodríguez, 2009; Roulin *et al.*, 2011; Simons *et al.*, 2012).

Las relaciones con ornamentos acromáticos, en cambio, han suscitado menor interés por considerarse poco costosas de producir.

En el caso de los ornamentos basados en pigmentos carotenoides, su expresión se ha relacionado con el estado oxidativo de los individuos ya que dichos pigmentos han de conseguirse por la dieta –siendo, por tanto, un recurso limitado-, podrían poseer propiedades antioxidantes (Pérez-Rodríguez, 2009) y además podrían ser muy susceptibles a la acción de los ERONs, que alterarían su estructura y sus propiedades cromáticas (Hill, 1992). En el caso de las señales melánicas, su expresión se ha vinculado al estrés oxidativo debido a que para la formación de melaninas oscuras (eumelaninas) se necesitan niveles bajos de glutatión (GSH), un potente antioxidante endógeno (Galván & Alonso-Alvarez, 2008, 2009).

Sin embargo, algunos ornamentos del plumaje están compuestos de áreas de plumas blancas, sin melanizar, que parecen no precisar pigmentos ni una nanoestructura ordenada determinada para su producción (Prum *et al.*, 1999). Es por este motivo por el que se consideran señales no costosas en términos de asignación de recursos, aunque se ha visto que tienen un elevado coste de mantenimiento, siendo más sensibles a la abrasión y a la degradación por parte de bacterias y ectoparásitos (ej. Barrowclough & Sibley, 1980; Kose *et al.*, 1999; Ruiz-De-Castañeda *et a.,* 2012).

Figura 3. *Ornamentos acromáticos del plumaje en el Papamoscas cerrojillo. A la izquierda macho con gran mancha en la frente. A la derecha bandas alares en la hembra.*

Así, en algunas especies del género *Ficedula* se han encontrado asociaciones entre el tamaño de la mancha blanca que presentan los machos (Figura 3) y algunas hembras en la frente con varios parámetros de estrés oxidativo (Markó *et al.*, 2011; Moreno *et al.*, 2011a, 2013a), lo que sugiere que estas áreas despigmentadas proporcionan información o implican ciertos costes relacionados, directa o indirectamente, con el estado oxidativo de los individuos (Morales *et al.*, 2008; Moreno *et al.*, 2011a, 2013a, 2013b; Costantini, 2014).

5. INVERSIÓN REPRODUCTIVA Y ESTRÉS OXIDATIVO

Como ya se ha comentado, una de las principales asunciones en los modelos de evolución de las estrategias vitales es que una elevada asignación de recursos/energía a una función no puede llevarse a cabo sin desviar esos recursos/energía desde otra función. Un ejemplo clásico de estos compromisos es el coste asociado a la reproducción, donde una elevada inversión reproductiva puede suponer una disminución de la subsiguiente fecundidad y futura supervivencia (Stearns, 1992). Los individuos en período reproductor deben optimizar esos compromisos a los que deben hacer frente y de este modo, ajustar el esfuerzo parental en función de su propia capacidad y de las circunstancias ambientales (Costantini, 2014). Unos de los factores considerados tradicionalmente como nexo entre inversión reproductiva y supervivencia es la elevada demanda energética requerida durante la reproducción (ej. Nilsson, 2002). Por ello, el estrés oxidativo ha recibido una considerable atención recientemente como mecanismo fisiológico subyacente a los costes ligados a la reproducción (Metcalfe & Monaghan, 2013).

5.1. El estrés oxidativo como coste y limitación en la reproducción

Cualquier fase del ciclo reproductor es susceptible de provocar un aumento en los niveles de estrés oxidativo (Metcalfe & Alonso-Alvarez, 2010). En aves se ha relacionado el tamaño y calidad de la puesta con el estado oxidativo de las hembras (Bertrand *et al.*, 2006; Alonso-Alvarez *et al.*, 2010). Además, el esfuerzo

parental durante la fase de alimentación de los polluelos también puede generar estrés oxidativo (Metcalfe & Alonso-Alvarez, 2010). Por ejemplo, se ha evidenciado que las tasas de cebas en aves están relacionadas con mayores tasas metabólicas (Moreno *et al.*, 2001; Nilsson, 2002) y, como consecuencia de este elevado metabolismo, un incremento en la producción de moléculas pro-oxidantes y radicales libres puede causar un aumento del estrés oxidativo (Von Schantz *et al.*, 1999). Algunos estudios experimentales en los que se manipuló el esfuerzo reproductor mediante un aumento de la pollada, también han reflejado estos costes, con efectos negativos sobre la resistencia al estrés oxidativo o la actividad antioxidante de los padres (Alonso-Alvarez *et al.*, 2004; Wiersma *et al.*, 2004).

Sin embargo, el signo de las asociaciones existentes entre reproducción y estado oxidativo puede variar entre distintas fases del ciclo reproductor, ya que el estrés oxidativo, además de ser un coste derivado de la inversión reproductiva, también puede actuar como un factor limitante para la reproducción (Bize *et al.*, 2008; Stier *et al.*, 2012). Por ejemplo, en ratones se ha visto que aquellas hembras con niveles bajos de daño oxidativo previos a la reproducción tienen mayores tamaños de camada que aquellas hembras con niveles más elevados (Stier *et al.*, 2012). De manera similar en aves, las hembras de estornino pinto (*Sturnus vulgaris*) con mayores niveles de daño oxidativo durante la incubación tienen un menor éxito reproductor (Costantini, 2014). Además, las asociaciones entre esfuerzo parental y estrés oxidativo pueden ser sexo-específicas, lo que sugiere que machos y hembras pueden desarrollar estrategias diferentes para hacer frente a esa inversión (Alsonso-Alvarez *et al.*, 2006; Costantini, 2008).

5.2. Compromiso entre reproducción y envejecimiento

El envejecimiento es un proceso complejo de progresivo deterioro debido a una acumulación de daños dentro del organismo, a nivel molecular, celular y orgánico (Kirkwood, 2005). La tasa de envejecimiento está ajustada evolutivamente a la capacidad de sobrevivir a factores extrínsecos del ambiente y viene determinada por la inversión del organismo en procesos de mantenimiento. Invertir en reproducción puede acelerar el envejecimiento, resultando generalmente en una reducción del potencial reproductivo y supervivencia futuros. En este sentido, el

estrés oxidativo puede constituir un vínculo entre esa inversión reproductiva y sus consecuencias sobre la eficacia biológica (Bauch *et al.*, 2013). Cuando la inversión reproductiva excede niveles sostenibles para los individuos parentales, los costes asociados pueden verse reflejados en un envejecimiento y senescencia acelerados (Badás *et al.*, 2015).

Desde principios de la década de los noventa se ha asociado el envejecimiento a un acortamiento en la longitud de los telómeros (Harley, 1990), lo que sugiere que la reducción de los telómeros podría ser un importante mecanismo responsable de la senescencia replicativa. La longitud telomérica se acorta en cada evento de replicación, y cuando esa pérdida de longitud supera un punto crítico se produce una detención permanente en el ciclo celular a través del proceso de senescencia celular (Haussmann & Marchetto, 2010). La tasa de acortamiento de telómeros no es, sin embargo, constante, sino que varía a lo largo de la vida y se acelera cuando el organismo está expuesto a ciertos factores, como el estrés oxidativo (Von Zglinicki, 2002; Epel *et al.*, 2004). Harman estableció por primera vez en 1957 que el envejecimiento es debido a la acción oxidante de los radicales libres (*"free radical theory of ageing"*). A nivel evolutivo, la inversión en el mantenimiento de los telómeros, por ejemplo a través de la telomerasa, varía en función de la capacidad de sobrevivir a los riesgos y costes de la vida. Como ya se ha comentado, durante la reproducción se pueden alcanzar niveles elevados de estrés oxidativo (ej. Alonso-Alvarez *et al.*, 2004; Metcalfe & Alonso-Alvarez, 2010). Además, algunos estudios han demostrado en aves que eventos reproductivos costosos pueden tener un impacto negativo sobre la dinámica telomérica en individuos reproductores (Reichert *et al.*, 2014), y en polluelos en desarrollo (Nettle *et al.*, 2013; Boonekamp *et al.*, 2014; Herborn *et al.*, 2014). Así, entender la relación que existe entre el estrés oxidativo y el acortamiento de los telómeros puede desentrañar los mecanismos que subyacen a las estrategias vitales determinadas por los compromisos existentes entre reproducción y envejecimiento.

6. OBJETIVOS Y ESTRUCTURA DE LA TESIS

Como se ha visto a lo largo de esta introducción, el estrés oxidativo ha sido considerado como un mecanismo próximo universal implicado en los compromisos vitales a los que se enfrentan los organismos a lo largo de la vida, gobernando así su diseño evolutivo. Por ello, el objetivo general de esta tesis es determinar, desde un punto de vista ecológico-evolutivo, el papel del estrés oxidativo en relación a las distintas estrategias vitales desarrolladas por las aves. En general, se han estudiado las adaptaciones fisiológicas, en relación al estrés oxidativo, a los diferentes compromisos a los que se enfrentan las aves a lo largo de su ciclo vital, particularmente durante el desarrollo y el período reproductor, dos etapas de especial vulnerabilidad por la alta demanda energética y fisiológica que conllevan. Para ello el modelo de estudio elegido ha sido el Papamoscas cerrojillo, un ave paseriforme de pequeño tamaño que anida en oquedades, bien adaptada al uso de cajas-nido durante el período de cría.

La tesis se estructura siguiendo la historia vital de los individuos. Para ello en los Capítulos I, II y III se estudia la influencia del ambiente de nidificación y la implicación de la carga de ectoparásitos del nido sobre la condición física y el estado oxidativo de polluelos y hembras reproductoras. El Capítulo IV analiza diferentes factores intrínsecos y ambientales que influyen sobre el estado oxidativo de los polluelos en desarrollo. A continuación, en el Capítulo V se exploran las relaciones existentes en diferentes etapas de la reproducción entre el estrés oxidativo de los individuos adultos y la expresión de ornamentos del plumaje. Por último, en el Capítulo VI se aborda la asociación entre la dinámica telomérica y los niveles de estrés oxidativo en machos y hembras adultos, además de explorar como se relacionan la longitud y el acortamiento de los telómeros de los padres con el estado oxidativo de sus polluelos.

Siguiendo el orden de los capítulos, los objetivos concretos son:

1. Debido a que el material de los nidos antiguos puede contener y/o atraer a más ectoparásitos que el material fresco, el primer objetivo de la tesis ha sido conocer de forma experimental el efecto que tiene la reutilización de los

nidos sobre la abundancia de ectoparásitos y su implicación sobre los parámetros de reproducción y la condición física de los pollos (Capítulo I).

2. Para estudiar el efecto de la carga de parásitos del nido sobre sus hospedadores aviares se emplean diferentes métodos físicos o químicos. Dado que el uso de insecticidas para este fin puede tener efectos no controlados en el ambiente de nidificación, un segundo objetivo fue testar de forma experimental el efecto de un insecticida de uso frecuente en este tipo de estudios sobre el éxito reproductor, el cuidado parental, la condición física y los niveles totales de glutatión (tGSH), un potente antioxidante endógeno implicado en la detoxificación de xenobióticos, de las hembras adultas y los polluelos (Capítulo II).

3. Puesto que la presencia de ectoparásitos del nido puede suponer costes para los hospedadores, constituyendo una importante fuerza evolutiva para las aves que anidan en oquedades, el tercer objetivo de esta tesis ha sido el de examinar experimentalmente los efectos de dichos parásitos sobre el estrés oxidativo y la condición física de polluelos en desarrollo y sus madres durante la incubación y tras la eclosión, períodos de mayor vulnerabilidad por el íntimo contacto que los individuos tienen con el material del nido (Capítulo III).

4. El estado oxidativo experimentado durante el desarrollo temprano puede tener consecuencias a medio y largo plazo sobre la eficacia biológica. Sin embargo las causas que lo modulan no están muy estudiadas en polluelos por lo que el cuarto objetivo de esta tesis es explorar de una forma más exhaustiva la contribución de factores genéticos, parentales y ambientales sobre los niveles de estrés oxidativo de juveniles en el nido (Capítulo IV).

5. Un aspecto clave para entender el papel potencial que los ornamentos del plumaje tienen en la comunicación de la condición de los individuos, es el estudio de los costes asociados a su producción y mantenimiento expresados en términos de estrés oxidativo. Para ello, aquí se ha querido examinar la interacción que existe entre la expresión de estos caracteres sexuales y el estado oxidativo de machos y hembras adultas a lo largo del

ciclo reproductor y su relación con la inversión reproductiva y el esfuerzo parental (Capítulo V).

6. Invertir en reproducción puede acelerar el envejecimiento y la senescencia, teniendo el estrés oxidativo un papel mediador en estas interacciones. El último objetivo de esta tesis ha sido el de investigar las asociaciones existentes entre el estado oxidativo y la dinámica telomérica en individuos adultos durante dos eventos reproductores y cómo el envejecimiento de estos afecta a su descendencia en términos de estrés oxidativo (Capítulo VI).

A continuación de los capítulos se incluye una Síntesis general en la que se discuten los resultados más relevantes y se enumeran las principales Conclusiones que se extraen de esta tesis.

7. ÁREA DE ESTUDIO

El seguimiento de las poblaciones de estudio para los trabajos presentados en esta tesis se ha llevado a cabo durante las primaveras de 2011 a 2014. Para el experimento de reutilización de nidos realizado en el Capítulo I se siguió una población de Papamoscas cerrojillo en un bosque cerca del municipio de Lozoya, situado al norte de la Comunidad de Madrid. La zona de estudio se sitúa a una altitud de unos 1500 m y en ella hay colocadas 100 cajas-nido en las que se han venido realizando diferentes estudios de aves trogloditas desde el año 2001 (Moreno *et al*., 2008, 2010). Los estudios recogidos en los Capítulos II a VI se han llevado a cabo en un bosque situado en la localidad de Valsaín a unos 1200 m de altitud, dentro del municipio de Real Sitio de San Ildefonso, provincia de Segovia (Figura 4). En esta área se han estudiado las poblaciones de aves desde 1991 (Sanz *et al.*, 2003) y actualmente hay colocadas 570 cajas-nido (Lambrechts *et al*., 2010).

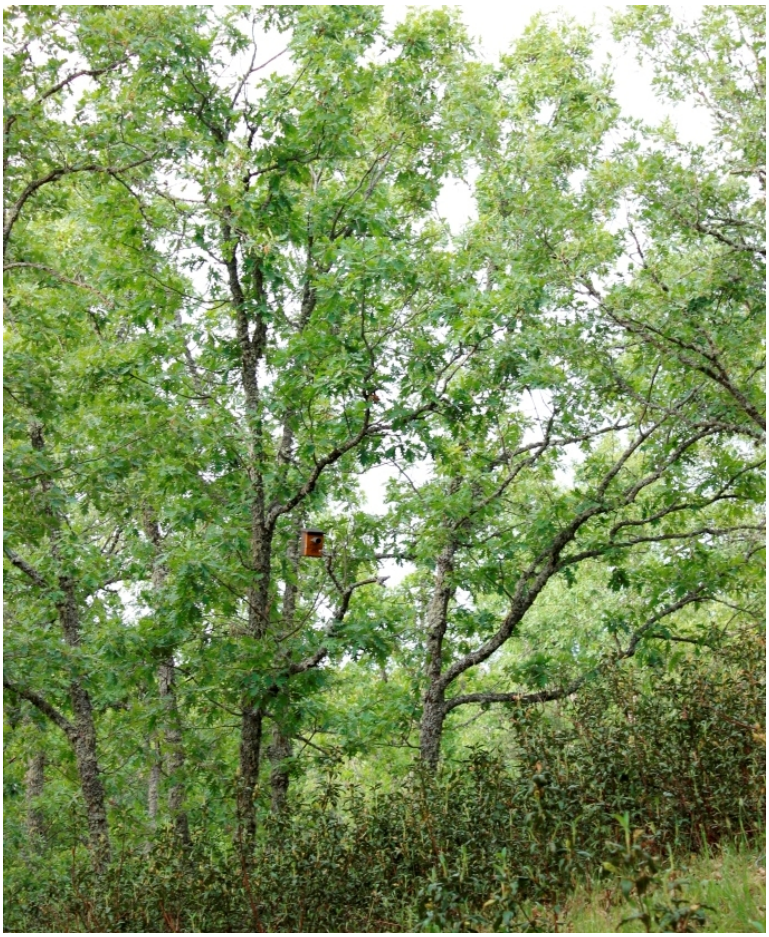

Figura 4. *Zona de estudio situada en los Montes de Valsaín (Segovia).*

Ambas zonas de estudio se conforman de bosque montano compuesto principalmente por roble melojo (*Quercus pyrenaica*) y en el que aparecen de forma más dispersa algunos pinos silvestres (*Pinus silvestris*) y jarales (*Cistus laurifolius*). Se trata de árboles bastante jóvenes en los que escasean los ejemplares de gran porte donde encontrar agujeros apropiados para la cría. Las especies de aves que comúnmente utilizan las cajas-nido durante la época reproductora son el Papamoscas cerrojillo, el Herrerillo común (*Cyanistes caeruleus*), el Carbonero común (*Parus major*), el Trepador azul (*Sitta europaea*) y el Gorrión chillón (*Petronia petronia*).

Aquí se realizan rutinariamente actividades de seguimiento desde las primeras fases de construcción de los nidos, hasta que los polluelos abandonan la caja. Así, se anotan la fecha y tamaño de puesta, la fecha de eclosión y los tamaños de nidada tras la eclosión y antes de que los polluelos abandonen el nido.

Generalmente todas las cajas se limpian, eliminando los restos de material viejo antes de cada temporada reproductora (pero ver Capítulo I).

8. MODELO DE ESTUDIO: EL PAPAMOSCAS CERROJILLO

Las aves constituyen un modelo idóneo para el estudio del papel del estrés oxidativo en la historia vital. Como ya se ha comentado, son organismos de vida larga, con lentas tasas de envejecimiento en comparación con otros animales de su mismo tamaño. Además, a pesar de presentar un metabolismo y un gasto energético bastante más elevados, parecen tener mecanismos moleculares únicos para hacer frente a los posibles daños en tejidos y a la senescencia fisiológica que pueden derivar de esos rasgos (Costantini, 2008).

El Papamoscas cerrojillo es un ave paseriforme de pequeño tamaño (12 - 13 g) que se reproduce en áreas boscosas de la región Paleártica (Lundberg & Alatalo. 1992). En España, durante la reproducción, se distribuye de forma más o menos fragmentada, ya que se restringe a zonas montañosas (Merino & Potti, 1997). Es un ave migradora que pasa el resto del año en las áreas de invernada al oeste de África (Lundberg & Alatalo, 1992). Es una especie bien adaptada a criar en cajas-nido, lo que hace de ella un popular modelo de estudio. Además, la facilidad para capturar a los adultos en las cajas-nido y el fácil manejo de los individuos suponen una enorme ventaja en estudios fisiológicos y ecológicos en condiciones naturales.

En la Península Ibérica cría la subespecie *Ficedula hypoleuca iberiae*, algo más pequeña y con manchas alares y frontales de mayor tamaño en los machos (Mullarney *et al.*, 2003). En primavera, durante el período nupcial existen marcadas diferencias de color entre ambos sexos. Los machos presentan un plumaje con un alto contraste entre el negro del dorso y la cola y el plumaje blanco de la parte inferior del cuerpo. Las hembras sin embargo, son de color pardo-grisáceo en el dorso y blanco en la zona inferior. Ambos sexos presentan bandas alares blancas, de mayor tamaño en los machos (Cantarero *et al*., 2017) Además, todos los machos y algunas hembras poseen una característica mancha blanca en la frente

(Potti, 1993; Morales *et al*., 2007). Los juveniles difieren bastante de los adultos, con un plumaje pardo, moteado de blanco ocre.

Las hembras comienzan a reproducirse antes que los machos, ya en el primer o segundo año de edad, mientras que estos últimos lo hacen a partir del segundo o tercer año (Potti & Montalvo, 1991). Las hembras no parecen mostrar indicios de senescencia reproductiva hasta los 5 años (Sanz & Moreno, 2000) y aunque escasos, hay datos de algunas parejas criando a los 7 años de edad (Potti, 2000).

Aunque es la hembra la que generalmente se encarga de la construcción del nido, se ha observado que un 25% de los machos también aportan material al nido (Martínez-De la Puente *et al*., 2009). A finales de mayo suele comenzar el período de puesta, con tamaños que varían de 4 a 7 huevos (Figura 5). Los huevos son de color azul verdoso debido principalmente a la elevada concentración de biliverdina que posee la cáscara en la poblaciones ibéricas (Morales *et al.,* 2013). Este pigmento además se ha visto que posee importantes propiedades fisiológicas entre las cuales cabe destacar su gran potencial antioxidante (Moreno & Osorno, 2003; Morales *et al*., 2008). La fase de incubación tiene una duración aproximada de 14 días. La hembra incuba sola y es parcialmente alimentada por el macho durante este período (Moreno et al., 2011b; Cantarero *et al*., 2014).

Figura 5. *Nido con huevos de Papamoscas cerrojillo dentro de una caja-nido (Foto: A. Cantarero).*

Tras la eclosión de los huevos la hembra pasa alrededor de una semana empollando a los recién nacidos (Sanz & Moreno, 1995). Los polluelos permanecen en el nido entre 15 y 19 días aproximadamente, tiempo durante el cual son alimentados por ambos padres.

9. MEDIDAS DE ESTRÉS OXIDATIVO Y ENVEJECIMIENTO CELULAR

Actualmente hay multitud de análisis para medir todos los componentes del estrés oxidativo. Sin embargo, esta batería de métodos está en constante revisión, existiendo aún un debate inacabado sobre qué medidas son más adecuadas para estimar el estado oxidativo en el organismo (Monaghan *et al*., 2009). Aunque estos métodos incluyen distintos tipos de tejido para llevar a cabo los análisis, en estudios ecológico-evolutivos sobre especies silvestres en condiciones naturales como los que se presentan en esta tesis, es importante seleccionar técnicas poco invasivas y que supongan un coste mínimo para los individuos, por lo que el uso de pequeñas cantidades de sangre para este fin se considera una buena opción (Stier *et al.*, 2015).

Por otro lado, una selección apropiada de los biomarcadores del estado oxidativo también es importante. La cuantificación de los niveles de un único componente del estrés oxidativo es insuficiente (Hõrak & Cohen, 2010; Selman *et al.*, 2012), por lo que generalmente se requiere la combinación de diferentes medidas como pueden ser la cuantificación de niveles de radicales libres, la estimación de defensas antioxidantes y la determinación de algunos tipos de daño oxidativo (Metcalfe & Monaghan, 2013). En el caso de los radicales libres, la estimación de niveles de ERO suele tener dificultades derivadas de su alta reactividad y su corto tiempo de vida, por lo que se requieren técnicas complejas para su cuantificación, con una aplicabilidad limitada en estudios en este campo (Monaghan *et al*., 2009; Pérez-Rodríguez *et al*., 2015). Por otro lado, las defensas antioxidantes comprenden una amplia gama de compuestos de diferente naturaleza química, y los niveles de antioxidantes individuales pueden no reflejar la fuerza de tales defensas. La capacidad antioxidante no es el resultado de la simple adición de la actividad de compuestos antioxidantes individuales, ya que se

producen interacciones sinérgicas y antagonistas entre ellos (Monaghan *et al*., 2009; Pérez-Rodríguez, 2009). Es por esto que una aproximación al estudio de defensas antioxidantes sea la aplicación de metodologías que tratan de estimar la capacidad total antioxidante (Cohen *et al*., 2007). Puesto que el daño oxidativo es el resultado del desequilibrio entre esos dos componentes del estrés oxidativo, también se pueden emplear técnicas de medición de subproductos derivados de la oxidación producida en diferentes biomoléculas (ADN, proteínas o lípidos) que sirvan como biomarcadores de daño oxidativo. Por ejemplo, entre las técnicas que miden daño en lípidos se encuentra la cuantificación de hidroperóxidos lipídicos ("*reactive oxygen metabolites*" o ROMs), frecuentemente empleada en estudios eco-fisiológicos por su facilidad de uso, aunque podría tener limitaciones derivadas de su baja especificidad (Kilk *et al*., 2014; Pérez-Rodríguez *et al*., 2015; pero ver también Costantini, 2016). El método más usado tanto en biología como en estudios médicos es la determinación de niveles de malondialdehído (MDA), un subproducto final de la cascada de peroxidación lipídica (Halliwell & Gutteridge, 2007; Mateos & Bravo, 2007; Monaghan *et al*., 2009).

Esta tesis incluye varias de las medidas de las que se dispone actualmente. El tejido empleado es la sangre, de la cual se han utilizado tanto el plasma como la fracción celular. Debido al pequeño tamaño de nuestra especie modelo, la extracción de sangre (sin superar el 10% del volumen estimado según el peso del animal) se ha realizado en la vena braquial (Figura 6), minimizando el posible riesgo de lesiones a los individuos durante la manipulación. Los métodos han sido seleccionados principalmente por su potencial informativo del estado oxidativo, y para ello se han obtenido muestras pequeñas, de alrededor de unos 100 µl de sangre, por lo que también se ha tratado de optimizar al máximo el uso de sangre para abarcar varias medidas combinadas.

Figura 6. *Extracción de sangre de la vena braquial en hembra adulta de Papamoscas cerrojillo.*

En los Capítulos III a VI de esta tesis se ha estimado la capacidad antioxidante total en el plasma ("*total antioxidant status*" o TAS; Miller *et al*., 1993) mediante un técnica colorimétrica dinámica en la que se mide la capacidad de la muestra para hacer frente al ataque por radicales libres (Cohen *et al*., 2007; Monaghan *et al*., 2009). Es importante considerar que esta medida puede verse afectada por los niveles de ácido úrico circulantes debido a sus propiedades antioxidantes (Cohen *et al*., 2007; Hõrak *et al*., 2007; Pérez-Rodríguez *et al*., 2008). Una variación en los niveles de ácido úrico, sin embargo, puede ser una consecuencia indirecta del catabolismo de aminoácidos. Por ello se recomienda corregir la medida de capacidad antioxidante por la concentración de esta molécula (Cohen *et al*., 2007), tal y como se ha llevado a cabo en esta tesis.

Otra molécula antioxidante que hemos cuantificado a lo largo de esta tesis (Capítulos II a VI) ha sido el glutatión, frecuentemente considerado como uno de los antioxidantes intracelulares más importantes y abundantes del organismo, presente en todas las células eucariotas (Wu *et al*., 2004; Isaksson *et al*., 2011). Su síntesis es endógena, aunque requiere de aminoácidos que se obtienen a través de la dieta (Wu *et al*., 2004; Isaksson *et al*., 2011). Durante su actividad antioxidante, el glutatión se oxida (GSSG) para posteriormente reciclarse volviendo

a su forma reducida de nuevo, de modo que el índice entre ambas moléculas es bastante constante (Halliwell & Gutteridge, 2007). Así, la medida de los niveles de tGSH en eritrocitos nos proporciona información sobre su funcionalidad y sobre el estado redox a nivel celular (Halliwell & Gutteridge, 2007).

Por último, en los Capítulos III a VI se ha estimado el daño oxidativo en lípidos cuantificando los niveles de MDA en plasma mediante cromatografía líquida de alta eficacia ("*high performance liquid chromatography*" o HPLC) con detector de fluorescencia. Puesto que los lípidos circulantes en sangre, relacionados con la ingesta reciente o la movilización desde tejidos de reserva (Halliwell & Gutteridge, 2007), son vulnerables a la oxidación, la cantidad presente en las muestras puede influir sobre los niveles de MDA (Pérez-Rodríguez *et al*., 2015). Controlar los niveles de MDA por la cantidad de lípidos presentes en el plasma puede modular la información que esta medida aporta (Romero-Haro & Alonso-Alvarez, 2014). Por este motivo en esta tesis también se han medido los niveles de triglicéridos en plasma como un indicador de la cantidad de lípidos circulantes.

Como biomarcador de senescencia celular en el Capítulo VI se ha analizado la longitud de los telómeros. La dinámica telomérica puede reflejar el estrés pasado y predecir la subsecuente morbilidad y mortalidad, independientemente de la edad cronológica. Existen diversos métodos para medir la longitud de los telómeros (revisión en Nussey *et al*., 2014), entre los cuales en esta tesis se han utilizado técnicas basadas en la reacción en cadena de la polimerasa cuantitativa ("*quantitative polymerase chain reaction*" o qPCR). Mediante este método se puede determinar la cantidad relativa de secuencias teloméricas existentes, usando pequeñas cantidades de ADN y con un mínimo tiempo de manejo de muestras. Una limitación a tener en cuenta es que con esta técnica también se amplifican secuencias intersticiales no teloméricas, las cuales pueden variar entre especies y entre individuos, pudiendo dar lugar a dificultades a la hora de encontrar patrones (Nussey *et al*., 2014). Sin embargo, se han encontrado buenas correlaciones entre las medidas de longitud telomérica que incluyen y excluyen las repeticiones intersticiales mediante el análisis de fragmentos de restricción de telómeros ("*telomere restriction fragment*" o TRF; Foote *et al*., 2013) y entre los análisis de TRF y qPCR (Criscuolo *et al*., 2011). No obstante, puede resultar especialmente interesante analizar, además de la longitud en un determinado momento, la

dinámica telomérica, midiendo los cambios de longitud que se producen a nivel individual con el tiempo, tal y como se ha realizado también en el Capítulo VI de esta tesis.

Información más detallada de las medidas y técnicas empleadas en los estudios que componen esta tesis se encuentra desarrollada en cada capítulo.

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SÓLO ALGUNAS POBLACIONES DE ECTOPARÁSITOS RESULTAN AFECTADAS POR LA REUTILIZACIÓN DE NIDOS

López-Arrabé, J., Cantarero, A., González-Braojos, S., Ruiz-De-Castañeda, R. & Moreno, J. 2012. Only some ectoparasite populations are affected by nest re-use: an experimental study on pied flycatchers. *Ardeola*, 59:253-266

RESUMEN

La reutilización de nidos en aves es poco común, sin embargo las cavidades más apropiadas para la cría pueden ser escasas, por lo que aves trogloditas, a menudo, pueden volver a ocupar las que utilizaron en temporadas anteriores conteniendo nidos viejos. Debido a que el material de los nidos antiguos puede contener y/o atraer a más ectoparásitos que el material fresco, es importante conocer el efecto que tiene la reutilización de los nidos sobre la abundancia de las distintas especies de ectoparásitos con diferentes virulencias y su implicación sobre los parámetros de reproducción y sobre la condición de los pollos. Se han estudiado las implicaciones de la reutilización de los nidos en una población reproductora de papamoscas cerrojillo (*Ficedula hypoleuca*) del centro de España, ofreciéndoles para anidar cajas-nido con material viejo y cajas-nido limpias. Hemos seguido la actividad de cría desde las primeras etapas de la construcción del nido hasta el vuelo de los pollos, siendo los nidos posteriormente retirados para estimar la abundancia de ectoparásitos. Las tasas de ocupación fueron similares para ambos tratamientos. Hemos encontrado que la abundancia de moscas hematófagas y pulgas son significativamente mayores en nidos con material viejo que en nuevos, pero la abundancia de ácaros, los ectoparásitos más virulentos de nuestra población hospedadora de estudio, no se han visto afectados por la presencia de material viejo en el nido. El crecimiento de los pollos con respecto a la longitud del tarso y al peso no se vio afectado por la reutilización de los nidos, aunque la longitud del ala estuvo marginalmente y negativamente afectada por la reutilización de nido. No hubo asociación entre la abundancia de ectoparásitos y el tamaño y la condición de los pollos. Estos resultados cuestionan la generalización de que existen mayores infestaciones en nidos con material viejo en la que se han basado ciertas críticas a la utilización de cajas-nido en estos estudios.

ABSTRACT

Nest re-use in birds is rare but since appropriate cavities may be scarce cavity nesting birds may often re-use those that were occupied in previous seasons. Old nest material may contain and/or attract more ectoparasites than fresh material. Therefore it is important to understand the effects of nest re-use on the abundance of different ectoparasite species of different virulence and their implication for breeding parameters and nestling condition. We studied the consequences of nest re use in a population of pied flycatchers (*Ficedula hypoleuca*) breeding in central Spain by offering them both nest-boxes with old nest material and cleaned nest-boxes. We monitored breeding activity from the early stages of nest construction until fledging, and then finally removed nests to estimate ectoparasite abundances. Occupation rates were similar for both treatments. We found that blowfly and flea abundances were significantly higher in old nests than in new nests, but the abundance of mites, the most virulent ectoparasites on our host study population, was not affected by the presence of old nest material. Nestling growth with respect to tarsus length and mass was not affected by nest re-use although wing length was marginally and significantly reduced by nest re-use. There was no association between ectoparasite abundance and nestling growth and condition. These results question the generality of assumed higher infestations in re-used nests, on which a certain critique of nest-box studies has been based.

Keywords: breeding, cavity nesting, *Ficedula hypoleuca*, haematofagous arthropods, nestling condition, old nest

INTRODUCTION

Although nest re-use in birds is rare (Hansell, 2000), appropriate cavities may be scarce and secondary cavity nesters may often re-use those that were occupied in previous seasons (Loye & Carroll, 1998; Tomás *et al.*, 2007). Most secondary cavity nesting birds build their nests on top of old nest material and only a few species clean their nest sites by removing old nest material (Pacejka & Thompson, 1996; Mazgajski, 2007). Whether the accumulation of old material is beneficial or detrimental to nesting birds remains controversial. While the presence of old nest material may reduce the costs of nest building behaviour (Møller, 1990; Mappes *et al.*, 1994; Moreno *et al.*, 2010), serve as a cue of nest site quality (Erckmann *et al.*, 1990; Mappes *et al.*, 1994) or allow olfactory masking of nestling odours to predators (Petit *et al.*, 2002), old nest material can also negatively affect breeding performance not only by making the cavity shallower but also by creating good conditions for ectoparasite development (Mazgajski, 2007). Thus, secondary cavity nesters are confronted with a trade-off between the costs and benefits of nest re-use. Some secondary cavity nesters have been shown to be sensitive to costs associated with the increased presence of nest-dwelling ectoparasites (Stanback & Dervan, 2001). Thus, some species have been shown to discriminate between high and low infestation levels in used nests and to choose accordingly (Oppliger *et al.*, 1994; Rendell & Verbeek, 1996a). Nest-dwelling ectoparasites may result in costs for nestlings of cavity nesters in terms of compromised growth and condition before fledging. If nest re-use implies increased prevalence and intensities of ectoparasite infestation, adaptations of cavity nesting birds to reduce the costs of infestation should be apparent (e.g. Richner *et al.*, 1993; Merino & Potti, 1995b; Allander, 1998; Heeb *et al.*, 1998, 2000; Fitze *et al.*, 2004; Tomás *et al.*, 2008; Moreno *et al.*, 2008a, 2009; Martínez-De la Puente *et al.*, 2010). Furthermore, the costs of nest re-use may also involve adults through the increased attraction of ectoparasitic vectors of blood parasitaemias (Tomás *et al.*, 2007).

Certain studies have revealed associations of old nest material with an increased abundance of certain types of ectoparasites. Most studies of nest re-use have been conducted on species breeding in artificial nest-boxes (but see Wesolowski, 2011 for a critique of this approach). Thus, several studies have

focused on the nesting preference of passerines by comparing occupation of cleaned nest-boxes and nest-boxes containing old nests (e.g. Orell *et al.,* 1993; Merino & Potti, 1995a; Olsson & Allander, 1995; Pacejka & Thompson, 1996), and establishing the implications of this choice on breeding success and nestling growth (e.g. Mappes *et al.*, 1994; Johnson, 1996; Tomás *et al.*, 2007; García-Navas *et al.*, 2008).

The most common ectoparasites of different virulence in nests of Iberian pied flycatcher (*Ficedula hypoleuca*) populations are mites (*Dermanyssus gallinoides*), fleas (*Ceratophyllus gallinae*) and the larvae of blowflies (*Protocalliphora azurea*). Mites have the strongest effect on nestling growth and mortality in some populations (Merino & Potti, 1996, 1998; Potti *et al.*, 1999, 2002; Potti, 2007; Lobato *et al.*, 2005, 2008; Moreno *et al.*, 1999, 2008b, 2009; Martínez-De la Puente *et al.*, 2009, 2010) and their prevalence, often exceeding 60%, is usually higher than for blowflies and fleas (40-50%) (Merino & Potti, 1995b; Moreno *et al.,* 2009; Tomás *et al.*, 2007, 2012). It therefore seems important to study the differential effects of nest re-use on the abundance of these three ectoparasitic species in relation to their virulence (Møller & Erritzøe, 1996; Pacejka *et al.*, 1998).

In the context of these studies, we aimed to test the effects of nest re-use on the abundance of the three common nest ectoparasite species, as well as on occupation rates and on breeding parameters (laying date, clutch size, hatching date, brood size at fledging) and nestling condition in pied flycatcher broods in a population breeding in central Spain. We explored the consequences of nest re-use by offering pied flycatchers nest-boxes with old nest material (from now on called re-used nests) belonging to nests of either pied flycatchers or blue tits (*Cyanistes caeruleus*) and cleaned empty nest-boxes without any nest material, in which to breed. We explored:

1. Whether re-used nests contained more ectoparasites than new nests, since part of their populations might overwinter in the nest material waiting for new hosts in the following season (Tomás *et al.*, 2007).

2. Whether ectoparasite abundance was affected by presence of old nest material.

3. Whether nestling growth in re-used nests was reduced, given the potential effects of nest-dwelling ectoparasites.

4. Whether flycatcher breeding is delayed and reproductive success reduced in reused nests.

METHODS

Study species and locality

The study was conducted in spring 2011 in a montane deciduous Pyrenean oak (*Quercus pyrenaica*) forest near Lozoya, central Spain (at 1500 m elevation), where long-term studies on cavity-nesting birds have been ongoing since 2001 (Moreno *et al.*, 2008a, 2010, 2011). In the study area, there are 100 wooden nest-boxes (for nest-box design, placement and manipulation, see appendix in Lambrechts *et al.*, 2010) occupied by pied flycatchers, blue tits and great tits, species that do not usually clean out nest boxes before nest-building. We followed breeding activity from the early stages of nest construction to fledging in nest-boxes occupied by pied flycatchers. These are small (12-13 g), hole-nesting, insectivorous passerines that readily breed in artificial nest-boxes and are thus frequently used as model species in avian ecology studies (Lundberg & Alatalo, 1992). Flycatchers in the study area start breeding in the second week of May, laying clutches of 4-7 eggs that are incubated for 14-15 days. On average six chicks fledge per nest, at the age of 17 days.

Blowfly larvae are parasites that feed intermittently on the blood of nestlings and in the meantime dwell in the nest material. Blowflies have long life cycles and their larvae pupate 9-14 days after hatching, so they can complete only one generation before chicks fledge (Bennett & Whitworth, 1991; Remeš & Krist, 2005). Flea larvae are not haematophagous but the adults need blood to produce eggs (Tripet & Richner, 1997). Therefore, the number of larvae in nests indicates the fecundity of adult fleas (Eeva *et al.*, 1994). Fleas can produce two generations in nests per season (Harper *et al.*, 1992). In mites both adult and some nymphal stages are haematophagous. They have short generation times and can reach the

adult stage in a few days, producing several generations and allowing the build-up of very large populations, with detrimental effects on host reproductive success (Merino & Potti, 1995b). All three ectoparasite types have been reported for both pied flycatchers and blue tits, although mites are usually most prevalent in flycatcher nests (see Introduction), whereas blowflies tend to show a higher prevalence in tit nests (around 80%; Merino & Potti, 1995b, 1996; Moreno *et al.*, 2008b, 2009; Tomás *et al.*, 2007, 2012).

Nest re-use experiment and data collection

After the 2010 breeding season, 37 nest-boxes containing nests of pied flycatchers and blue tits were not cleaned-out as usual after each breeding season, and 63 were cleaned. The nest-boxes were checked daily from early April (April 1 = day 1) to determine the start of nest construction and the amount of nest material collected, and breeding data, including laying date, clutch size and hatching date, were ascertained. Nestlings were measured at the age of 13 days (hatching day = age one) and mean measurements for each nest were later analysed. For each nestling the tarsal length was measured with a digital calliper to the nearest 0.01 mm, body mass was obtained with a Pesola® spring balance (precision 0.25 g), and wing length was measured with a stopped ruler. Chicks were ringed with numbered aluminium rings.

In order to establish ectoparasite abundances, nests were removed in sealed plastic bags soon after nestlings fledged and conserved in a cold room in the laboratory. They were later subjected to arthropod removal in Berlese funnels for 48 hours. The funnels were equipped with 60 W light bulbs and nest associates were driven into jars attached to the bottom of the funnels containing about 100 ml of a 96% ethanol solution. The entire content of the jars was examined by taking samples, which were spread in a 90 mm diameter Petri dish to get a homogeneous distribution of the parasites collected. The Petri dish had a squared paper (area = 36 cm^2) base divided into nine sub-squares. Three of the sub-squares inside the square and two of the four areas within the Petri dish but outside the square were searched for ectoparasites using a stereoscopic microscope (Olympus SZX2-ILLK). The number of parasites (mites, fleas and flea larvae) in the dish was calculated as

the number counted in the three squares multiplied by three plus twice the number counted in the two areas external to the squares. The Petri dish was then cleaned and the process repeated until the entire jar content had been processed. The separate counts were then added to obtain an estimate of the total number of mites and fleas in the jar (Merino & Potti, 1995b; Moreno *et al.*, 2009). Blowfly puparia in the nest material were counted by nest separation into different components. Nests were subsequently weighed on an electronic balance with 0.1 g precision.

Statistical analyses

Statistical analyses were conducted using STATISTICA (version 7.0, StatSoft, Inc.). The effects of nest type on breeding parameters were tested with ANOVA (laying and hatching date) and Kruskal-Wallis tests (clutch and brood size) depending on the distribution of the dependent variable. As the distributions of blowfly abundances could not be normalised through any transformation, nonparametric tests were used for this variable. Mite and flea abundances were successfully normalised through logarithmic transformations and were analysed with general linear models (GLM). We first tested whether the material of old nests (moss in nests constructed by blue tits, or leaves and grass in nests constructed by flycatchers, Moreno *et al.*, 2009) affected ectoparasite abundances, breeding parameters or nestling biometric variables. Since there were no significant effects (Table 1), both types of material were pooled into a single category of re-used nests when testing their effects on ectoparasite abundances or on nestling measurements, for hatching date and brood size at the age of 13 days. We also correlated nest mass with abundances of the different ectoparasites separately for new and re-used nests.

GLM analyses were used to check the effects of ectoparasites or nest type (new or re-used nests) on nestling biometric variables near fledging (tarsus length, wing length and body mass), controlling for hatching date and brood size at the age of 13 days.

Table 1. *Results of Kruskal-Wallis tests and GLM analyses for differences in ectoparasite abundances, breeding data and nestling biometrical variables (mean ± SE) in re-used nests built with different material (numbers of nests are in parentheses).*

	Moss (Tit nests)	*Grass (Flycatcher)*	*Statistic*	*p*
Ectoparasites				
Blowflies	15.44 ± 5.38(9)	4.50 ± 2.08(12)	$H_1 = 2.535$	0.111
Mites	355.89 ± 202.17(9)	417.25 ± 171.27(12)	$F_{1,19} = 0.079$	0.782
Fleas	279.22 ± 84.61(9)	212.42 ± 69.02(12)	$F_{1,19} = 0.256$	0.618
Nestling variables				
Tarsus length	17.44 ± 0.31(9)	17.15 ± 0.58(12)	$F_{1,19} = 1.785$	0.197
Body mass	13.89 ± 0.86(9)	13.73 ± 1.13(12)	$F_{1,19} = 0.131$	0.722
Wing length	44.47 ± 1.84(9)	43.00 ± 2.72(12)	$F_{1,19} = 1.943$	0.179
Breeding data				
Laying date	43.44 ± 3.17(9)	43.67 ± 3.02(12)	$F_{1,19} = 0.027$	0.872
Hatching date	62.67 ± 2.78(9)	63.27 ± 2.72(11)	$F_{1,18} = 0.240$	0.629
Clutch size	6.11 ± 0.33(9)	5.83± 0.58(12)	$H_1 = 1.658$	0.198
Brood size	5.78 ± 0.83(9)	5.27 ± 0.79(11)	$H_1 = 2.647$	0.104

RESULTS

A total of 55 nest-boxes were occupied by pied flycatchers. Of these 21 contained old nests from 2010, 12 of which had belonged to pied flycatchers and nine to blue tits, on which the flycatchers built a new nest-cup on top of the old nest material. The other 34 breeding pairs nested in cleaned nest-boxes. Occupation rates were similar for both treatments (53.97% for new nests and 56.76% for re-used nests). Ectoparasites were present in all studied nests. Specifically, mites and fleas were found in 54 nests (98.2%) and blowflies in 21 nests (38.2%). Breeding parameters were not significantly affected by nest type (GLM analyses: laying date: $F_{1, 53} < 0.001$, $p = 0.989$; hatching date: $F_{1, 52} = 1.513$, $p = 0.224$; Kruskal-Wallis tests: clutch size: $H_1 = 1.923$, $p = 0.166$; or brood size: $H_1 = 1.674$, $p = 0.196$). Nest mass was significantly higher in re-used than in new nests (GLM analysis: $F_{1, 53} = 97.526$, $p < 0.001$).

Table 2. Results of GLM analyses for effects of nest type on mite and flea abundances, controlling for brood size and hatching date (minimal models are selected by backward elimination of non-significant terms).

	Parameter	*SE*	*Df*	*F*	*P*	*Adjusted R²*
Mites						
Full model			48	0.887	0.455	-0.007
Hatching date	-0.063	0.077	1	0.674	0.416	
Brood size	-0.173	0.291	1	0.352	0.556	
Nest type	0.321	0.258	1	1.552	0.219	
Fleas						
Full model			48	3.085	0.036*	0.109
Hatching date	-0.119	0.064	1	3.512	0.067	
Brood size	-0.119	0.242	1	0.243	0.624	
Nest type	-0.552	0.214	1	6.660	0.013*	
Minimal model			51	4.736	0.013*	0.123
Hatching date	-0.135	0.059	1	5.095	0.028*	
Nest type	-0.509	0.209	1	5.962	0.018*	

* Significant difference ($\alpha = 0.05$)

Table 3. Results of GLM analyses for effects of nest type on nestling measurements, controlling for brood size and hatching date (minimal models are selected by backward elimination of non-significant terms).

	Parameter	*SE*	*Df*	*F*	*P*	*Adjusted R²*
Body mass						
Full model			48	2.543	0.067	0.083
Hatching date	-0.059	0.032	1	3.493	0.068	
Brood size	0.137	0.120	1	1.302	0.259	
Nest type	0.094	0106	1	0.784	0.380	
Minimal model			52	6.590	0.013*	0.095
Hatching date	-0.079	0.031	1	6.590	0.013*	
Tarsus length						
Full model			48	0.250	0.861	-0.046
Hatching date	0.007	0.018	1	0.139	0.710	
Brood size	0.046	0.067	1	0.468	0.497	
Nest type	-0.029	0.059	1	0.239	0.627	
Wing length						
Full model			48	2.089	0.114	0.060
Hatching date	-0.124	0.104	1	1.420	0.239	
Brood size	0.453	0.394	1	1.322	0.256	
Nest type	0.454	0.348	1	1.702	0.198	
Minimal model			53	3.975	0.051	0.052
Nest type	0.648	0.325	1	3.975	0.051	

* Significant difference ($\alpha = 0.05$)

Blowfly abundance was significantly higher in re-used nests than in new nests (Kruskal-Wallis test: $H_1 = 5.898$, $p = 0.015$; Figure 1.a) and did not correlate significantly with hatching date (Spearman correlation: $rs = 0.149$, $p = 0.280$, $N = 54$), brood size (Spearman correlation: $rs = 0.097$, $p = 0.489$, $N = 53$) or nest mass (Spearman correlations: new nests: $rs = 0.049$, $p = 0.785$, $N = 34$; old nests: $rs = 0.164$, $p = 0.476$, $N = 21$). Flea abundance was not affected by nest mass (Spearman correlation: new nests: $rs = 0.061$, $p = 0.730$, $N = 34$; re-used nests: $rs = 0.015$, $p = 0.946$, $N = 21$), but was significantly higher in re-used than in new nests when controlled for hatching date and brood size (Table 2; Figure 1.b). Nest type, controlled for hatching date and brood size, showed no significant effect on mite abundance (Table 2; Figure 1.c). Nest mass showed a negative association with mite abundance in re-used nests (Spearman correlation: $rs = -0.480$, $p = 0.028$, $N = 21$), while this effect was positive for new nests (Spearman correlation: $rs = 0.367$, $p = 0.033$, $N = 34$).

Nest re-use showed a marginally significant effect on wing length when controlled for hatching date and brood size, while there were no effects on mass and tarsus length (Table 3). Ectoparasite abundances were not related to nestling biometric measurements or body mass, when controlled for hatching date and brood size (Table 4).

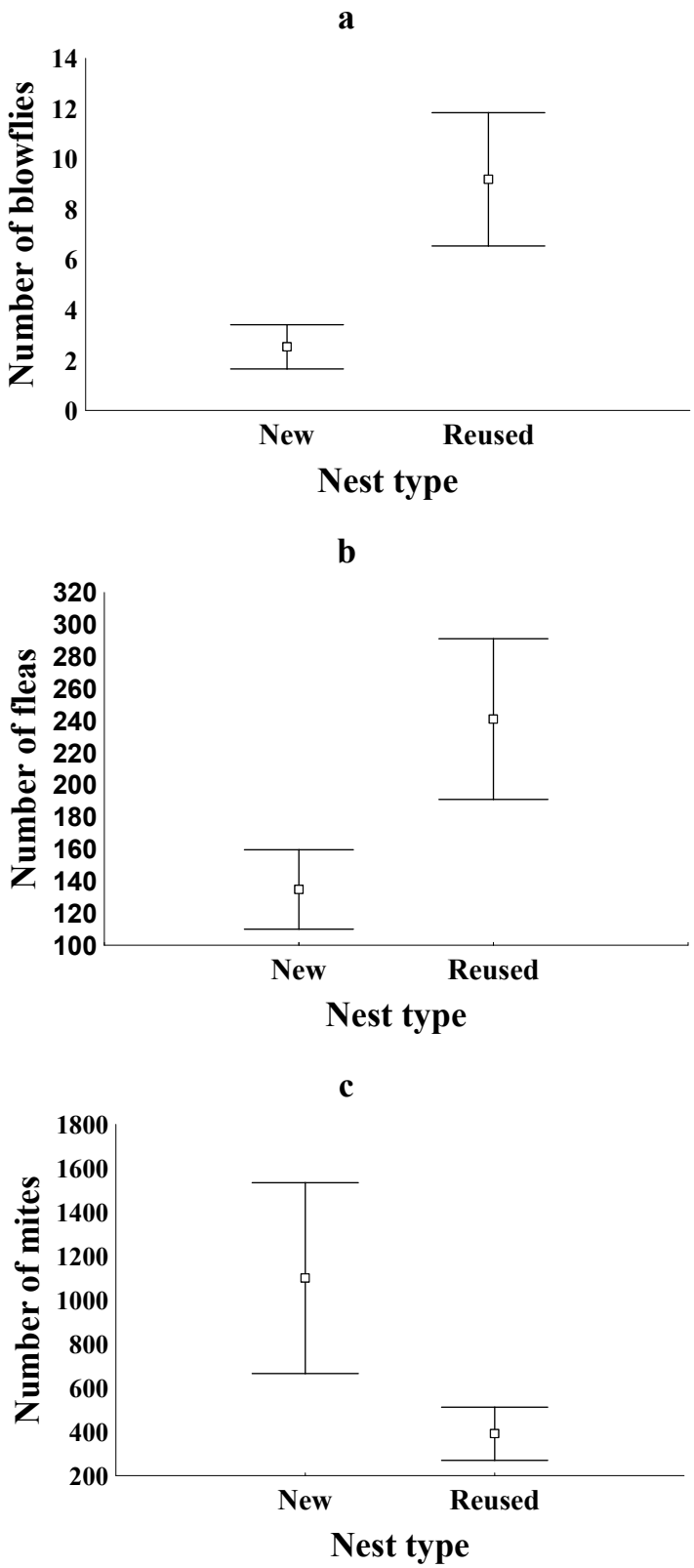

Figure 1. Mean ± SE number of (a) blowflies, (b) fleas and (c) mites in each type of nest.

Table 4. *Results of GLM analyses for effects of ectoparasite abundances on nestling linear measurements and mass, controlling for brood size and hatching date (minimal models are selected by backward elimination of non-significant terms).*

	Parameter	*SE*	*Df*	*F*	*P*	*Adjusted R^2*
Body mass						
Full model			46	1.781	0.136	0.071
Hatching date	-0.043	0.035	1	1.529	0.222	
Brood size	0.160	0.122	1	1.728	0.195	
Blowflies	-0.013	0.011	1	1.490	0.228	
Mites	<0.001	<0.001	1	0.254	0.616	
Fleas	0.001	0.001	1	1.156	0.288	
Minimal model			52	6.590	0.013*	0.095
Hatching date	-0.079	0.031	1	6.590	0.013*	
Tarsus length						
Full model			46	0.247	0.939	-0.079
Hatching date	0.012	0.019	1	0.362	0.550	
Brood size	0.043	0.069	1	0.396	0532	
Blowflies	-0.001	0.006	1	0.054	0.818	
Mites	-0.001	<0.001	1	0.013	0.909	
Fleas	<0.001	<0.001	1	0.591	0.446	
Wing length						
Full model			46	1.094	0.377	0.009
Hatching date	-0.179	0.117	1	2.309	0.135	
Brood size	0.547	0.406	1	1.809	0.185	
Blowflies	-0.002	0.037	1	0.004	0.951	
Fleas	-0.001	<0.001	1	0.053	0.819	
Mites	-0.002	0.002	1	1.031	0.315	

* Significant difference ($\alpha = 0.05$)

DISCUSSION

We have here conducted an experimental study of nest re-use by Iberian pied flycatchers. Our predictions, based on the literature, were only partly confirmed since mites were not more abundant in re-used nests, in contrast to fleas and blowflies. Although some studies have shown that infestations of the three types of ectoparasites have negative effects on pied flycatcher nestling condition in the short or long term (Potti, 2008) and on mortality in the nest (Merino & Potti, 1995b), mites are the ectoparasites with the strongest effects on nestling growth and survival in some populations (Merino & Potti, 1996, 1998; Potti *et al.*, 1999, 2002; Potti, 2007; Lobato *et al.*, 2005, 2008; Moreno *et al.*, 1999, 2008b, 2009;

Martínez-De la Puente *et al.*, 2009, 2010) including our own study population (J. Moreno, unpubl.). The lack of effects of nest re-use on the impact of the locally most virulent ectoparasites could explain the absence of marked associations of old nests with reduced nestling growth and survival in our study. This casts some doubt on the generality of the tenet that nest-box cleaning after each breeding season eliminates strong negative selective influences on avian life histories (Møller, 1990). We will discuss these results in turn.

The prevalence and abundance of blowflies were higher in old nests. Given that blowflies are thought to overwinter only rarely within the nest material (Bennett & Whitworth, 1991), we should expect that numbers of blowflies in nest-boxes with and without old nests should not differ (Johnson, 1996). Alternatively, more intense odour cues in nest-boxes with old nests may facilitate their detection by adult blowflies (Tomás *et al.*, 2007). In the case of flea abundances, removal of old nests by researchers might affect their populations, since flea populations are known to overwinter in nest-boxes (Rendell & Verbeek, 1996b; Rytkönen *et al.*, 1998; Mazgajski, 2007; Tomás *et al.*, 2007).

However, there was no detectable effect of nest re-use on mite abundance. Mites are the most virulent ectoparasites of Iberian pied flycatcher populations (Merino & Potti, 1995b, 1996, 1998; Potti *et al.*, 1999, 2002; Potti, 2007; Lobato *et al.*, 2005, 2008; Moreno *et al.*, 1999, 2008b, 2009; Martínez-De la Puente *et al.*, 2009, 2010). Nevertheless, mites did not affect nestling growth and survival in obvious ways in our study. Rendell & Verbeek (1996b) have shown a positive correlation between mite abundance and nest volume (although with high interannual variability), whereas in our study this association was negative in re-used nests and positive in new nests. The negative association of mite abundance with nest mass in reused nests suggests that old nest materials are inimical to mite proliferation, which could be due to there being a higher bacterial load in re-used nests than in new nests (González-Braojos *et al.*, 2012). This may explain the lack of significant effects of nest re-use on mite abundances.

We have not found any association of ectoparasite abundances with pied flycatcher breeding variables or nestling condition. These results agree with some studies of nest re-use in which no effect of ectoparasite abundance on breeding parameters has been detected (Mappes *et al.*, 1994; Rytkönen *et al.*, 1998;

Mazgajski, 2007). Richner & Heeb (1995) suggested that clutch size could be related to the life-cycle length of ectoparasites. Thus, in the presence of short-cycle ectoparasites, females should lay a smaller clutch than in the presence of only long-cycle parasites. It is doubtful whether females in our population can predict the future infestation of their nests so far in advance and at a breeding stage during which they scarcely visit the nest-box, which may explain absence of associations of nest type with clutch size. Breeding in a nest-box containing an old nest may be advantageous if it reduces nest building effort (Møller, 1990; Mappes *et al.*, 1994; Tomás *et al.*, 2007). This reduction in effort, which has been estimated in our study population (Moreno *et al.*, 2008; Morales *et al.*, 2010), could compensate for deleterious effects of nest re-use. It may also explain the lack of associations between ectoparasite loads in re-used nests and reproductive success variables. Given that even mites had no effect on nestling growth and survival in our study, it is possible that conditions for breeding were especially favourable in the area and year of study, making detection of the effects of nest re-use difficult.

Our results contradict some of the studies of the effects of nest re-use on breeding success (Mazgajski, 2007; García-Navas *et al.*, 2008), although they agree with others (Mappes *et al.*, 1994; Johnson, 1996; Pacejka & Thompson, 1996; Blem *et al.*, 1999; Hauber, 2002; Tomás *et al.*, 2007). The absence of associations of nest re-use with reduced nestling growth and survival in the nest may be explained by the absence of effects of re-use on the most virulent ectoparasite in our study system. However, some studies have shown that females may increase their energetic investment in provisioning or sanitation activities to compensate for the negative effects of ectoparasites on nestling condition and survival (Merino *et al.*, 1998; Hurtrez-Boussès & Renaud, 2000). This suggests that demonstrating associations between nest re-use and the abundance of an ectoparasite is insufficient to permit conclusions regarding effects on nestling fitness. As we did not study parental breeding effort, we cannot exclude the possibility that parents compensated for deleterious effects of nest re-use. Parental compensation would shift the costs of nest re-use from offspring to parents. Furthermore, our experimental design did not control for effects of parental quality on nest-box choice and occupation. However, we did not find any evidence that parental quality differed between treatments, since laying date and clutch size were not related to nest re-use. Furthermore, if better quality parents preferred to breed in cleaned nest-boxes

to avoid infestations of fleas and blowflies, we should expect negative effects of nest re-use on nestling growth and survival as both parental quality and ectoparasite abundance would lead towards improved fitness in cleaned nest-boxes, which was not the case. Although nestling growth is related to subsequent recruitment probabilities (Lobato *et al.*, 2005; Moreno *et al.*, 2005), we must be cautious in making generalisations. Thus, when conditional effects are not directly visible, detrimental effects may still manifest themselves over the longer term (Van de Crommenacker *et al.*, 2012). In this context, further studies should address the links between parasitic infection and the physiological condition of nestlings.

To conclude, nest re-use affects positively the abundance of some ectoparasites but not others. In our host population, the nonaffected ectoparasite turns out to be the only one having consistently deleterious effects on nestling growth and mortality. This could be due to the absence of effects of nest reuse on reproductive success and nestling condition at fledging, although parental compensation for ectoparasite effects is also possible. In any case, our findings question the general assumption that old nests result in increased nest infestations, on which a certain critique of nest-box studies has been based. However, more subtle effects of increases in less virulent ectoparasites, and possible costs derived from increased parental effort, cannot be excluded and should be explored in future studies.

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EL TRATAMIENTO EXPERIMENTAL CON PIRETROIDES SUBESTIMA LOS EFECTOS DE LOS ECTOPARÁSITOS SOBRE LAS AVES QUE ANIDAN EN CAVIDADES DEBIDO A SU TOXICIDAD

López-Arrabé, J., Cantarero, A., Pérez-Rodríguez, L., Palma, A., & Moreno, J. 2014. Experimental pyrethroid treatment underestimates the effects of ectoparasites in cavity-nesting birds due to toxicity. *Ibis*, 156:606-614

RESUMEN

Los ectoparásitos que habitan en los nidos pueden suponer costes para los polluelos de aves que anidan en cavidades, en términos de crecimiento y condición limitados antes de abandonar el nido. La reducción o eliminación de los ectoparásitos para estudiar sus efectos en aves puede llevarse a cabo a través de métodos físicos, como los tratamientos térmicos, o mediante métodos químicos usando insecticidas. Los piretroides son los más utilizados, aunque algunos estudios han demostrado que pueden comprometer el desarrollo y la supervivencia futura de las aves. En este estudio, realizado en el centro de España, se analizaron las diferencias entre un grupo de nidos de (*Ficedula hypoleuca*) fumigados y un grupo tratado térmicamente, eliminando los ectoparásitos con ambos tratamientos. También comparamos estos nidos exentos de ectoparásitos con un grupo control con cargas naturales de ectoparásitos. Nuestro objetivo fue testar los posibles efectos de un insecticida basado en piretroides sobre el éxito reproductivo, los comportamientos de cuidado parental y la condición corporal de las hembras adultas y los polluelos. También se determinaron los efectos del tratamiento en un biomarcador bioquímico, el nivel total de glutatión (tGSH), implicado en la detoxificación de xenobióticos y considerado el antioxidante intracelular más importante. Aunque las variables de comportamiento no se vieron afectadas por el tratamiento, los resultados mostraron polluelos de 3 días de vida con menor peso y con tarsos y alas más cortos en polluelos volantones en nidos fumigados, junto con una reducción de los niveles de tGSH tanto en hembras como polluelos. El uso de piretroides en experimentos de reducción de la carga de ectoparásitos puede introducir una variabilidad sistemática no deseada asociada a su toxicidad, lo que puede llevar a una subestimación de los efectos de los ectoparásitos en los hospedadores aviares.

ABSTRACT

Nest-dwelling ectoparasites may result in costs for nestlings of cavity nesters in terms of compromised growth and condition before fledging. The reduction or elimination of nest ectoparasites to study their effects on avian hosts can be conducted through physical methods such as heat-treatment or through chemical methods using insecticides. Pyrethroids are the most frequently used of the latter, although some studies have shown that they may compromise the development and future survival of birds. In this study conducted in central Spain we analysed the differences between a group of fumigated Pied Flycatcher (*Ficedula hypoleuca*) nests and a heat-treated group, both rendered ectoparasite free by these treatments. We also compared these ectoparasite-free nests with a control group with natural ectoparasite loads. Our aim was to test the possible effects of a pyrethroid- based insecticide on reproductive success, parental care behaviours and body condition of adult females and nestlings. We also determined the effects of treatment on a biochemical biomarker, the total glutathione (tGSH) level, involved in detoxification of xenobiotics and considered the most important intracellular antioxidant. Although behavioural variables were not affected by treatment, results showed lighter 3-day old chicks and shorter tarsi and wings in nestlings shortly before fledging in fumigated nests, together with depletion of tGSH levels in both females and nestlings. Fumigation with pyrethroids in ectoparasite load reduction experiments may introduce undesired systematic variability associated with toxicity, leading to underestimation of the effects of ectoparasites on avian hosts.

Keywords: body condition, glutathione, heat treatment, insecticide, nest-dwelling parasites, Pied Flycatcher

INTRODUCTION

Avian cavity-nesting has been traditionally associated with selective pressures derived from benefits in terms of the thermal environment and the impact of nest predation (Hansell, 2000). Although protected from weather and relatively safe from predators, nesting cavities constitute microclimatically stable environments that may offer excellent breeding and growth conditions for bacteria, decomposers and detritivores due to the presence of faeces and food remains of breeding birds, and for ectoparasites that feed on blood, skin and feathers of avian hosts (Collias & Collias, 1984).

Parasites may be an important ecological and evolutionary factor affecting avian life histories and behaviour (Atkinson & Van Riper, 1991; Møller, 1997). Nest-dwelling ectoparasites may result in costs for nestlings of cavity nesters in terms of compromised growth and condition before fledging (Heeb *et al.*, 2000; Tomás *et al.*, 2008; Brommer *et al.*, 2011; Cantarero *et al.*, 2013a). They may also affect adult behavioural responses directed towards minimizing their negative effects (Christe *et al.*, 1996; Heeb *et al.*, 1998; Tripet *et al.*, 2002, Cantarero *et al.*, 2013a).

Effects of nest-dwelling ectoparasites on hosts are frequently studied through correlational approaches by quantifying directly the number of parasites in nests, or through experimental manipulations of ectoparasite loads (Moss & Camin, 1970). These manipulations can be accomplished by increasing ectoparasite loads, specifically by the addition of certain parasites to nests, or by reducing them. The reduction or elimination of nest ectoparasites can be conducted through physical methods (direct extraction of parasites, freezing or heat-treating nests) or through chemical methods using insecticides. In these experimental manipulations, it is common to compare a sample of treated nests and another of unmanipulated nests as a control group. The assumption behind these treatments is that only ectoparasites are affected by the manipulation and that other environmental variables remain unaffected, thereby allowing the effect of the parasites to be deduced from the results. Therefore, it is important to control undesirable effects arising from handling that would alter our results.

The most frequently used insecticides in such experiments are pyrethroids (Szep & Møller, 2000; Heylen *et al.*, 2009), highly active synthetic insecticides derived from natural pyrethrins (Vijverberg & Van den Bercken, 1990) produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum*) that constitute the majority of commercial household insecticides. Most of them are composed of permethrin, sometimes accompanied by tetramethrin, at concentrations below 0.5%. The International Programme on Chemical Safety of the World Health Organization included some pyrethroids in 1990 in its Environmental Health Criteria documents and described their effects as highly toxic to fish and other aquatic organisms but of low or very low toxicity to birds and mammals. However, some studies have demonstrated their negative effects on poultry by disrupting cellular function through adverse effects on the activities of enzymes that contribute to the detoxifying activity of glutathione (GSH) (Ezeji *et al.*, 2012), one of the most efficient cellular tools for detoxification of drugs and xenobiotics in general (Pompella *et al.*, 2003). Reported effects of pyrethroids include haematological and biochemical alterations and damage to tissues such as kidney and liver in avian, fish and mammalian species (Khan *et al.*, 2012 and references therein) and negative effects on GSH-related metabolism in rats (Otitoju & Onwurah, 2005).

These effects may compromise the development and future survival of some organisms. Altricial nestlings of cavity-nesting birds could be especially vulnerable to these risks given the closed nesting environment in which pyrethroids are released and their still underdeveloped detoxification mechanisms. In a previous study with the same set of nests as we heat-treated here, we demonstrated and discussed the negative effects that ectoparasites have on nestling body condition when compared with control nests (Cantarero *et al.*, 2013a). Here we assess the effects of insecticide treatment on variables commonly reported in ectoparasite manipulations, such as reproductive success, parental care behaviour, body condition of adult females, and development and condition of nestlings. We also assess the effects of pesticide exposure on a biochemical biomarker related to detoxification, the intracellular total glutathione level (tGSH). tGSH level is expected to decrease in the pyrethroid-exposed treatment due to the disruptive effect of the drug on GSH metabolism (Ezeji *et al.*, 2012). In accordance with the assumption that the use of insecticides, due to their toxicity, neutralizes the positive effects of reducing nest ectoparasite loads, our predictions were that the effects on nestling

condition and growth of the two most widely used treatments to reduce ectoparasite loads on nests (heat and insecticides) differ from each other, and that the fumigation-treated nests do not differ from controls with natural ectoparasite loads. To that end, we studied a breeding population of the Pied Flycatcher (*Ficedula hypoleuca*), a typical cavity-nester with high prevalence of ectoparasite infestations.

METHODS

General field methods

The study was conducted during the spring of 2012 in a montane deciduous forest of Pyrenean Oak (*Quercus pyrenaica*) in Valsaín, central Spain (40°54´N, 4°01´W; 1200 m elevation), where long-term studies on cavity-nesting birds have been ongoing since 1991 (see Sanz *et al.*, 2003 for general description). In the study area there are 552 nest-boxes (see Lambrechts *et al.*, 2010 for dimensions, structure and placement of nest-boxes) occupied by Pied Flycatchers, Great Tits (*Parus major*), Eurasian Nuthatches (*Sitta europaea*) and Blue Tits (*Cyanistes caeruleus*). We followed breeding activities from the early stages of nest construction to fledging in nest-boxes occupied by Pied Flycatchers. Egg-laying in this Pied Flycatcher population typically begins in late May, and the modal clutch size is six. The female incubates and broods alone (Moreno *et al.*, 2011). No brooding is observed after nestlings attain 7 days of age (Sanz & Moreno, 1995) and chicks usually fledge at the age of 17 days.

At the age of 3 days we weighed all nestlings in each brood together on a digital balance to the nearest 0.1 g to determine mean chick mass. On day 13 (hatching day = day 1), nestlings were ringed, weighed and measured. Body mass was obtained with a Pesola® spring balance (Pesola AG, Baar, Switzerland) to the nearest 0.25 g, tarsus length was measured with a digital calliper (precision 0.01 mm) and wing length with a stopped ruler. We took a blood sample of about 120 µl from the brachial vein that was collected in heparinized microcapillaries. Blood samples (N = 333) were stored in Eppendorf tubes in an ice-box until returning to

the laboratory on the same day. Plasma was separated from blood cells by centrifugation (10 min at 14000 g) and then both fractions were stored at -80 °C until analysed for assaying tGSH levels (see below).

Parents were captured in their nest-boxes with traps while feeding nestlings of 7-8 days, ringed if necessary or identified by their ring. Females (*N* = 66) were also measured and blood-sampled in the same way as nestlings.

During blood collection, some samples may suffer rupture of erythrocytes, possibly due to changes in pressure during extraction. Haemolysis could cause a possible efflux of intracellular molecules into the extracellular environment that could affect the results of analyses. Thus, we controlled for haemolysis levels in plasma samples by detecting visually the red colour of plasma that occurs as a consequence of release of haemoglobin from red blood cells, and marking samples on a gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). To minimize inter-observer variability only one person noted haemolysis degree.

Protocol of experimental reduction of ectoparasites

Of the 91 nest-boxes occupied by Pied Flycatchers, we selected birds whose laying date was between dates 45 and 50 (April 1 = day 1, mean ± SE laying date = 47.94 ± 0.18). We applied different methods to reduce ectoparasite loads in nests during the egg-laying period by randomly allocating nests to the following three treatments: (1) a heat-treated group (*N* = 19) using a microwave oven to reduce ectoparasite loads, (2) a fumigated group (*N* = 14) sprayed with an insecticide, and (3) an unmanipulated control group with natural ectoparasite loads (*N* = 33).

In the first treatment, nests were heat-treated for about 30 s at 750 W. This treatment ensured that experimental nests did not contain live arthropods when placed in the nest-box (Rendell & Verbeek, 1996), although some arthropods may colonize the nest material after the treatment. To avoid the loss of water during the heat treatment, the nests were placed into a hermetic plastic container. Furthermore, before returning the original nest, the flame from a butane jet torch lighter (Microtorch GT- 3000, Blazer Products, New York, USA) was passed across the walls of the nest-box to kill ectoparasites that might remain there. While the

original nests were removed from the nest-boxes for treatment (around 30 min), a fresh substitute nest was introduced into the nest-box (these nests had been collected in previous seasons after being abandoned prior to laying and kept frozen at 20 °C until use).

In the fumigated group, nests and nest-boxes were sprayed with a commercial pyrethroid-based insecticide (Itec Spray, Natural Granen SA, Belgium; 0.3% permethrin, 0.2% tetramethrin and 1% piperonyl butoxide) for about 5 s and then aerated for 30 s. Chicks were previously removed from the nests and kept in a container with a cotton base during treatment.

To prevent recurrence of ectoparasite colonization, a total of three repetitions of the treatments were made: (1) 7 days after clutch completion, (2) when nestlings were 2 days old and (3) when nestlings were 8 days old. Nests in the control group were visited on the same days and handled in a similar way to experimental ones but without applying any treatment.

Ectoparasite abundance estimation

The most common ectoparasites in nests of Iberian Pied Flycatcher populations are mites (*Dermanyssus gallinoides*), fleas (*Ceratophyllus gallinae*) and the larvae of blowflies (*Protocalliphora azurea*). Mites have the strongest effect on nestling growth and mortality in some populations (Merino & Potti, 1996, 1998; Potti *et al.*, 1999, 2002; Potti, 2007; Lobato *et al.*, 2005, 2008; Moreno *et al.*, 1999, 2008, 2009; Martínez-de la Puente *et al.*, 2009, 2010) and their prevalence, often exceeding 60%, is usually higher than for blowflies and fleas (40-50%) (Merino & Potti, 1995; Moreno *et al.*, 2009; Tomás *et al.*, 2007, 2012; Cantarero *et al.*, 2013b). Blowfly larvae are parasites that feed intermittently on the blood of nestlings and otherwise dwell in the nest material (Bennett & Whitworth, 1991; Remeš & Krist, 2005). Flea larvae are not haematophagous, but the adults need blood to produce eggs (Tripet & Richner, 1997). Therefore, the number of larvae in nests indicates the fecundity of adult fleas (Eeva *et al.*, 1994). In mites, both adult and some nymphal stages are haematophagous (Merino & Potti, 1995).

At 1 or 2 days after nestlings fledged (17 days after hatching), all nests were removed in sealed plastic bags and taken to the laboratory, where they were subjected to arthropod removal in Berlese funnels for 48 h until nests were thoroughly dried and no arthropods were moving in the nest material. Ectoparasite identification was made with the aid of a stereoscopic microscope (Olympus SZX7, Olympus Iberia, Barcelona, Spain); for arthropod collection and abundance estimations see Moreno *et al.* (2009).

Determination of tGSH levels

tGSH levels in red blood cells were determined according to Galván & Alonso-Alvarez (2008) with a few modifications. Red blood cell samples were diluted (1 : 20 w/v) and homogenized in a stock buffer (0.01 M phosphate-buffered saline and 0.02 M EDTA) using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK, USA) and mixed with an equal volume of 10% trichloroacetic acid. The mixture was vortexed three times for 5 s each bout within a 10-min period. The mixture was then centrifuged (2000 g, 15 min, 6 °C), and the supernatant was separated. Three working solutions were made up in a reaction buffer (125 mM Na-phosphate and 6.3 mM EDTA) as follows: (1) 0.3 mM NADPH, (2) 6 mM DTNB and (3) 50 U GSH reductase/ml. Solutions 1 and 2 were mixed at 7 : 1 volume. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). To 75 µl of sample (supernatant) we added 240 µl of the mixture of solutions 1 and 2. Then, 20 ml of solution 3 was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH from 0.5 to 0.031 mM. Only one 12-well row was used from the plate at a time. A subset of samples assayed in duplicate showed high repeatability ($R = 0.929$, $N = 44$, $p < 0.001$).

Behavioural data

At 2 and 8 days after the day of hatching of the young, we recorded nest activity inside nest-boxes for about 90 min with a cold white light (5 mm LED) powered by a 3 V battery and a camera (GoPro HD Hero1, Woodman Labs, San Mateo, CA, USA) mounted on the roof inside the nest-box. All films were recorded between 08:00 and 17:00 h (for more details see Cantarero *et al.*, 2013a, 2013b) and no differences between groups with respect to time of filming were found (general linear model (GLM) analyses: early nestling phase: $F_{2,63}$ = 2.056, p = 0.136; late nestling phase: $F_{2,63}$ = 1.517, p = 0.227).

From recordings during the early nestling phase we obtained hourly provisioning rates by males and females and brooding attendance, estimated as the proportion of time spent by the female inside the nest-box.

From recordings during the late nestling phase we obtained hourly provisioning rates by males and females.

Statistical analysis

Statistical analyses were conducted using STATISTICA (version 8.0; StatSoft, Inc., Tulsa, OK, USA). We first tested for success of treatments to reduce or eliminate ectoparasite loads on nests using Kruskal-Wallis tests and then for the relationship between treatment and breeding parameters in three groups with ANOVA (laying and hatching dates) or Kruskal-Wallis tests (clutch size) depending on the distribution of the dependent variable.

Brood sizes at days 3 and 13 and hatching (proportion of eggs that hatched) and fledging successes (proportion of hatched chicks that fledged) were analysed with Kruskal-Wallis tests. Mean body mass per chick when nestlings were 3 days old was analysed with GLM with treatment as fixed factor. All behavioural data and adult female body mass and tGSH levels were analysed in the same way. Nestling morphological and biochemical variables measured before fledging (day 13) were analysed with GLM including nest identity nested within treatment as random factor and treatment as fixed factor.

Although degree of haemolysis in blood samples of nestlings and females was not affected by treatment (Kruskal-Wallis test: females: $H_2 = 0.562$, $N = 63$, $p = 0.755$; nestlings: $H_2 = 0.246$, $N = 329$, $p = 0.884$), haemolysis may affect tGSH levels, thereby confounding interpretation of results. Thus, we controlled for this factor in all tGSH analyses.

These analyses were performed to test differences between three groups. Post-hoc analyses were performed when models were significant in order to explore pair wise comparisons of means by Fisher's least significant difference (LSD) tests.

RESULTS

Ectoparasite loads and breeding parameters

The three types of ectoparasites were greatly reduced or eliminated in heat-treated and fumigated groups in comparison with control nests (Table 1). The three treatments did not differ with respect to laying date, hatching date and clutch size (Table 1).

Reproductive success

The three treatments did not differ with respect to brood size (Kruskal-Wallis test: brood size at day 3: $H_2 = 0.657$, $N = 66$, $p = 0.720$; brood size at day 13: $H_2 = 1.902$, $N = 66$, $p = 0.386$), hatching success (Kruskal-Wallis test: $H_2 = 0.686$, $N = 66$, $p = 0.709$) or fledging success (Kruskal-Wallis test: $H_2 = 2.523$, $N = 66$, $p = 0.283$).

Parental care behaviours

Neither brooding attendance nor hourly provisioning rates by females and males at early nestling phase were affected by treatment, as were provisioning rates before fledging (all $p > 0.20$).

Table 1. *Differences in ectoparasite abundances and breeding variables (mean ± SE) between three treatments and results of Kruskal-Wallis and GLM tests.*

	Control	*Heat*	*Insecticide*	*Statistic*	*p*
Ectoparasites					
Blowflies	5.54±1.17	0.68±0.43	0.00±0.00	H = 22.221	<0.001*
Mites	3713.58±815.35	274.05±208.06	83.86±35.49	H = 25.006	<0.001*
Fleas	27.82±16.19	0.00±0.00	0.00±0.00	H = 11.504	0.003*
Breeding variables					
Laying date	48.15±0.25	47.37±0.34	48.21±0.39	F = 2.040	0.139
Hatching date	66.45±0.24	65.95±0.32	66.50±0.37	F = 1.000	0.387
Clutch size	5.67±0.11	5.84±0.15	5.57±0.18	H = 1.116	0.572

* Significant difference (α = 0.05)

Table 2. *Results of GLM analyses for effects of treatment in nestling morphological variables at early and late phases (treatment as fixed factor at day 3; nest nested within treatment as random factor and treatment as fixed factor at day 13) and in adult females body mass (treatment as fixed factor) after controlling by brood size and laying date.*

	Control	*Heat-treated*	*Fumigated*	*Statistic (F)*	*Df Residual*	*Partial η^2*	*Model p-value*	*Post-hoc p-values*		
								F-C	*F-H*	*C-H*
Nestlings day 3										
Mean body mass	3.36±0.10	3.76±0.13	3.23±0.15	4.453	63	0.124	0.015*	0.461	0.009*	0.016*
Nestlings day 13										
Body mass	13.97±0.05	14.16±0.06	14.12±0.08	0.342	258	0.011	0.712	-	-	-
Tarsus length	17.48±0.03	17.77±0.04	17.61±0.05	4.128	255	0.113	0.020*	0.093	0.022*	< 0.001*
Wing length	47.07±0.13	48.67±0.16	47.59±0.20	4.739	245	0.129	0.012*	0.398	< 0.001*	< 0.001*
Female										
Body mass	12.79±0.14	12.32±0.19	13.29±0.23	5.224	61	0.146	0.008*	0.085	0.009*	0.168

C = Control group; H = Heat-treated group; F = Fumigated group. * Significant difference (α = 0.05)

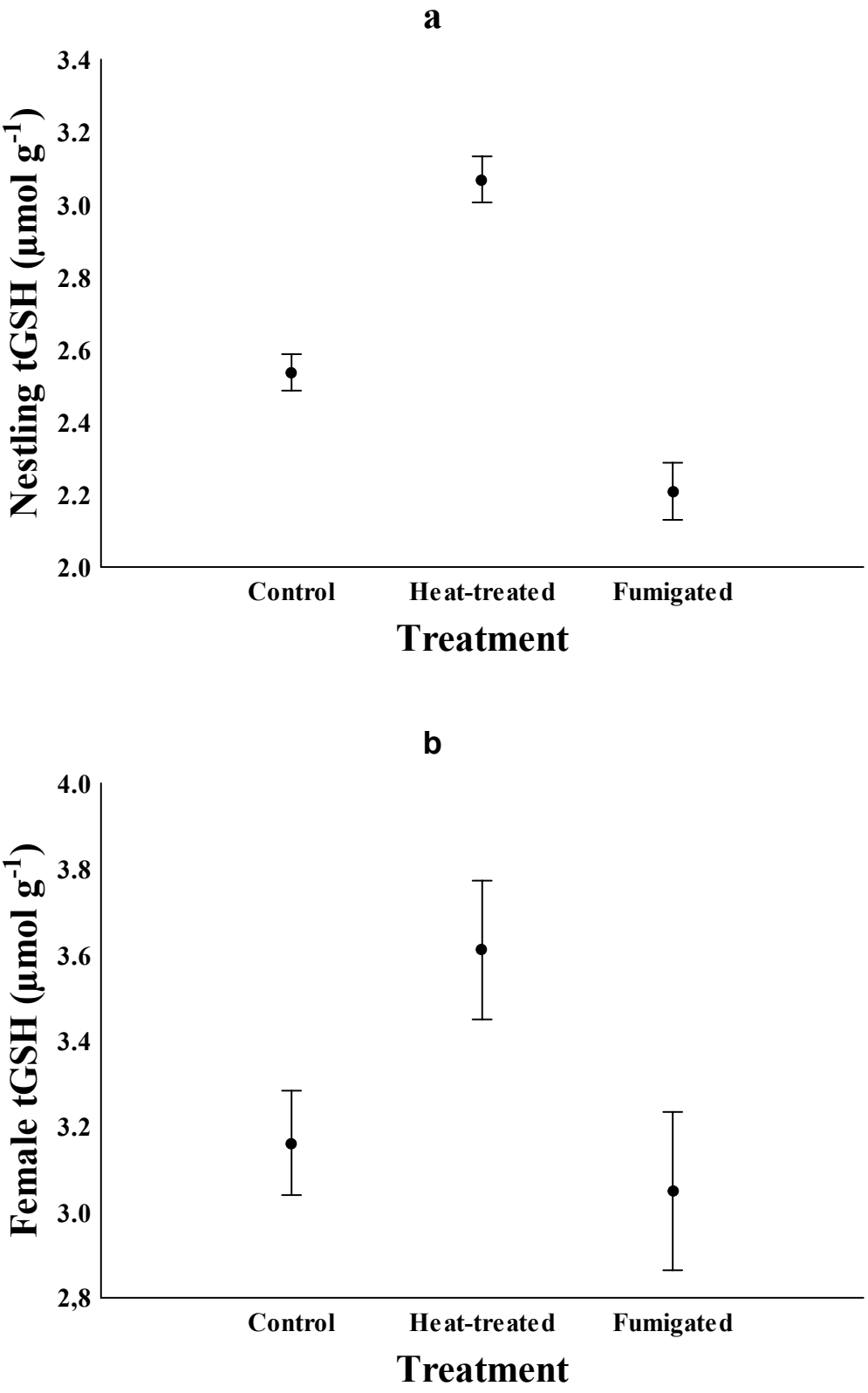

Figure 1. Mean ± SE tGSH levels in relation to treatment in (a) nestlings and (b) adult females.

Body condition

Mean body mass per chick on day 3 was significantly higher in the heat-treated group than in the fumigated nests (Table 2). At fledging, chicks in heat-treated nests had longer tarsi and wings than chicks in fumigated nests (Table 2). Nestling body mass at fledging was not different between ectoparasite reduction treatments (Table 2). No morphological parameter of females and nestlings differed between control and fumigated nests (Table 2). Female body mass was affected by treatment, being significantly higher in the fumigated than in the heat-treated group (Table 2).

tGSH levels

Nestlings showed differences in tGSH between treatments, with lower levels in fumigated nests (F = 11.272, N = 329, $p < 0.001$; pair-wise comparisons: all $p < 0.001$; Figure 1.a) after controlling by haemolysis score (F = 14.174, $p < 0.001$). Females showed significantly higher levels of tGSH in heat-treated nests than in the fumigated group (F = 3.334, N = 60, p = 0.043; pair-wise comparisons: control-fumigated: p = 0.722; fumigated-heat-treated: p = 0.022; control-heat-treated: p = 0.017; Figure 1.b) after controlling by haemolysis score (F = 6.309, p = 0.015).

DISCUSSION

We conducted an experimental study to test the effects of two different treatments to reduce nest ectoparasite loads on parental care and body condition of females and nestlings in an Iberian Pied Flycatcher population. Our results showed that both types of randomly applied treatments were effective in greatly reducing or eliminating ectoparasite loads, with both treatments being similarly efficient. Although parental care behaviours were not affected by treatments, results confirmed our initial hypothesis about the negative effects of pyrethroid-based insecticides, as expressed by lighter 3-day old chicks, reduced skeletal and

integumentary development shortly before fledging, and a reduction of tGSH, an important biomarker of redox status with an essential role in cellular detoxification.

Shortly before fledging, nestlings showed reduced tarsus and wing lengths in fumigated nests compared with heat-treated ones, without differences between fumigated and control nests. Thus, nestling growth was negatively affected by ectoparasites in the control group (Cantarero *et al.*, 2013a) and by the insecticide in fumigated nests. Tarsus length of Pied Flycatcher nestlings has been related to their recruitment probability (Alatalo & Lundberg, 1986), so these negative effects may affect the future fitness of nestlings. Wing development at fledging may also be crucial during the first post-fledging days (Nilsson & Gårdmark, 2001). The lack of an effect of treatment on nestling body mass may be due to favourable conditions for breeding during the year of study (largest body masses since 1991, Cantarero *et al.*, 2013a) affecting nestling mass more strongly than structural and integumentary growth.

Female body mass differed between treatments in the opposite direction to that predicted if toxicity impaired maternal condition. We have no convincing explanation for this striking result, although it may suggest a reallocation of maternal resources to survival instead of current reproduction where offspring have been affected by toxicity.

At the physiological level, GSH is a very important detoxifying agent, enabling the body to get rid of undesirable toxins and pollutants and playing an important role as an antioxidant and in detoxification and elimination of xenobiotics (pesticides) (Meister & Anderson, 1983; Otitoju & Onwurah, 2007). The depletion of tGSH in individuals exposed to pyrethroid-based insecticides could be explained in two non-mutually exclusive ways. On the one hand, GSH reacts with a large number and variety of foreign compounds with an electrophilic centre to form GSH conjugates. The interaction of foreign compounds with GSH may be spontaneous or catalysed by GSH S-transferases (Meister & Anderson, 1983). GSH-adducts formed after conjugation of electrophiles are then actively secreted from the cell, eventually resulting in the depletion of cellular GSH (Pompella *et al.*, 2003). Thus, reduction in GSH level is an indication that detoxification is taking place (Ezeji *et al.*, 2012). Alternatively, GSH operates in the reduction of the disulphide linkages of proteins and in the protection of cells against oxidative damage, effectively scavenging free

radicals and other reactive oxygen species (Meister, 1991; Wu *et al.*, 2004). At normal levels of oxidative stress there is essentially no net loss of GSH through oxidation (Griffith, 1999). However, if pro-oxidant levels increase sufficiently, GSH protects cells by reacting rapidly with peroxides and producing GSSG (glutathione disulphide, an oxidized form of GSH). Because GSSG is not taken up intact by cells but rather is degraded extracellularly, GSSG efflux from cells contributes to a net loss of intracellular GSH (Griffith, 1999; Wu *et al.*, 2004). Thus, the depletion of cellular tGSH levels in individuals from fumigated nests may indicate an increase in reactive oxygen and nitrogen species (RONS, and, as a consequence, an increase in oxidative status of individuals related to treatment (Kale *et al.*, 1999). In nestlings, the decrease in tGSH levels was even higher in fumigated nests than in controls, which may indicate that the insecticide has stronger adverse effects than ectoparasites during development. In the case of adult females, the depletion of intracellular tGSH suggests a direct negative effect on female physiology, probably suffered through direct exposure during the incubation and brooding phases, and not only due to the work overload associated with increased nestlings needs.

The depletion of tGSH shown in our results is in accordance with several studies based on pesticide effects (e.g. Della Morte *et al.*, 1994; Kale *et al.*, 1999; Ezeji *et al.*, 2012; Fetoui & Gdoura, 2012). Oxidative stress is a key cost that limits rates of growth in the wild (Hall *et al.*, 2010). An adequate availability of antioxidants has been shown to enhance both pre- and post-hatch growth, reduce susceptibility to pathogens, and increase the ability of chicks to withstand oxidative damage (Surai, 2002). Thus, a depletion in GSH levels, considered the most important intracellular antioxidant (Meister, 1991), might be involved in the trade-off between self-maintenance and growth rate of nestlings and between self-maintenance and breeding effort in adult individuals during nestling development phase.

We also demonstrated that the negative effects on nestling condition exposed to a pyrethroid-based insecticide were explained by its toxicity and not by changes in behaviours related to parental care, as these were not related to treatment. Our results suggest that the choice by researchers of method to reduce ectoparasite loads to test their effects on organisms could affect the conclusions derived from the experiments. This is the first study to our knowledge that shows

the different effects of two experimental methods used to reduce ectoparasite loads in avian nests. The use of pyrethroid insecticides can introduce in this type of experiment undesired systematic variability associated with toxicity that leads to underestimations of the effects of ectoparasites on avian hosts.

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LOS ECTOPARÁSITOS DEL NIDO REDUCEN LAS DEFENSAS ANTIOXIDANTES EN HEMBRAS Y POLLUELOS

(Foto: A. Cantarero)

López-Arrabé, J., Cantarero, A., Pérez-Rodríguez, L., Palma, A., Alonso-Alvarez, C., González-Braojos, S., & Moreno, J. 2015. Nest-dwelling ectoparasites reduce antioxidant defences in females and nestlings of a passerine: a field experiment. *Oecologia*, 179:29-41

RESUMEN

Los ectoparásitos pueden implicar costes en términos de estrés oxidativo provocado por una respuesta inflamatoria en los huéspedes. Los ectoparásitos además pueden resultar en costes para polluelos y hembras en período de incubación debido a la pérdida directa de nutrientes y a una reducción de la capacidad metabólica resultante de la actividad de alimentación del parásito. Estas respuestas pueden resultar en la producción de especies reactivas de oxígeno y nitrógeno que pueden inducir daño oxidativo en los tejidos del huésped. Nuestro objetivo fue examinar el efecto de los ectoparásitos en términos de estrés oxidativo en polluelos y hembras adultas en una población de Papamoscas cerrojillo (*Ficedula hypoleuca*). Manipulamos toda la comunidad de ectoparásitos del nido, reduciendo su carga mediante tratamiento térmico, comparando con un grupo control de nidos con cargas naturales. Un marcador de la capacidad antioxidante total (TAS) en plasma y los niveles totales de glutatión (tGSH) en los glóbulos rojos, además de un marcador de daño oxidativo en lípidos plasmáticos (malondialdehído; MDA) fueron medidos simultáneamente. Los niveles de tGSH fueron mayores en nidos tratados térmicamente que en los controles, tanto en hembras como en polluelos. Los valores de TAS fueron más elevados en las hembras de nidos experimentales. En polluelos hubo una correlación negativa entre TAS y MDA. Nuestro estudio apoya la hipótesis de que los ectoparásitos exponen a las aves que anidan en cavidades a un desafío oxidativo. Esto podría tener costes a largo plazo, comprometiendo la eficacia biológica de los individuos.

ABSTRACT

Ectoparasites may imply a cost in terms of oxidative stress provoked by inflammatory responses in hosts. Ectoparasites may also result in costs for nestlings and brooding females because of the direct loss of nutrients and reduced metabolic capacity resulting from parasite feeding activities. These responses may involve the production of reactive oxygen and nitrogen species that may induce oxidative damage in host tissues. Our goal was to examine the effect of ectoparasites in terms of oxidative stress for nestlings and adult females in a population of pied flycatchers (*Ficedula hypoleuca*). We manipulated the entire nest ectoparasite community by reducing ectoparasite loads in some nests through a heating treatment and compared them with a control group of nests with natural loads. A marker of total antioxidant capacity (TAS) in plasma and total levels of glutathione (tGSH) in red blood cells as well as a marker of oxidative damage in plasma lipids (malondialdehyde; MDA) were assessed simultaneously. Levels of tGSH were higher in heat-treated nests than in controls for both females and nestlings. Higher TAS values were observed in females from heat-treated nests. In nestlings there was a negative correlation between TAS and MDA. Our study supports the hypothesis that ectoparasites expose cavity-nesting birds to an oxidative challenge. This could be paid for in the long term, ultimately compromising individual fitness.

Keywords: Antioxidant status, glutathione, malondialdehyde, oxidative stress, Pied flycatcher

INTRODUCTION

Parasites and pathogens are important evolutionary forces (Atkinson & Van Riper, 1991; Møller, 1997) contributing to the emergence of different protection mechanisms such as behavioural defences, physical barriers (e.g. skin, scales, etc.) and the immune system (Hõrak *et al.*, 2006; Sepp *et al.*, 2012). The activation of some of these defences is energetically costly and this can play an important role in physiological and life history strategies of the host. In contrast to endoparasites that live in intimate contact with their host, ectoparasites are characterized by free-living stages whose key habitat is the surface of the host or their immediate environment. It has previously been shown that environmental stressors, such as parasite exposure and infection (Saino *et al.*, 2002; Costantini, 2008; Sorci & Faivre, 2009) can result in oxidative stress on hosts because of the resulting upregulation of the immune system, which is the main physiological defence mechanism against parasites (Zuk & Stoehr, 2002). In this context, we should consider that ectoparasites could be costly, at least in part, because they induce oxidative stress in their hosts, which is associated with long-term effects, including accelerated aging (e.g. Golden *et al.*, 2002; Metcalfe & Alonso-Alvarez, 2010). Oxidative stress is usually defined as the imbalance between the rate of production of reactive oxygen and nitrogen species (RONS) by the organism and state (levels, integrity or activity) of the antioxidant machinery (Halliwell & Gutteridge, 2007; Metcalfe & Alonso-Alvarez, 2010). Endogenous antioxidants, like uric acid or glutathione, are synthesized by the organism. Exogenous antioxidants, like vitamins A, C and E, and carotenoids, must be obtained from food (Halliwell & Gutteridge, 2007). When an imbalance in antioxidants occurs, it may lead to oxidative damage in important biomolecules (lipids, proteins and DNA), which could impair their functionality (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2007).

The effect of any type of parasite on oxidative stress is mediated by the immune response of the host. In the case of ectoparasites, small wounds are produced when they bite the host, through which an oral secretion is introduced into the skin tissues. These secretions have antigenic properties that induce an inflammatory response (Baron & Weintraub, 1987; Owen *et al.*, 2010). Different immune cells generate RONS in order to destroy pathogens (Halliwell & Gutteridge,

2007; Costantini & Møller, 2009; Sorci & Faivre, 2009). However, when uncontrolled, these RONS may also affect the host tissues, leading to oxidative stress (Sorci & Faivre, 2009). Furthermore, the induction of an immune response due to ectoparasite activity may also increase metabolic activity (Møller *et al.*, 1994; Demas *et al.*, 1997), thereby raising RONS levels, and hence, favour an imbalance in antioxidant status (Finkel & Holbrook, 2000). This, combined with an energetically costly adjustment of other physiological and behavioural traits (Richner *et al.*, 1993; Christe *et al.*, 1996; Cantarero *et al.*, 2013a), may also result in higher levels of tissue oxidative damage under stressful conditions (Von Schantz *et al.*, 1999; Van de Crommenacker *et al.*, 2012). In summary, oxidative stress can be considered as a proximate mechanism involved in the cost of parasitism, and is key to understanding life history tradeoffs between growth, reproduction and self-maintenance (Costantini, 2008; Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010; Metcalfe & Monaghan, 2013).

Our goal is to examine the effect of ectoparasites in terms of oxidative stress in a population of wild birds. Birds are suitable for the study of oxidative stress as they are generally long lived, in comparison to mammals of similar size, and they apparently have strategies to cope with a much higher metabolic rate and energy expenditure than mammals (Costantini, 2008). Bird-ectoparasite associations have also provided many influential examples of parasite-mediated evolution and ecology (Proctor & Owens, 2000). Many ectoparasite species live and reproduce within the nest material, feeding intermittently or continuously on hosts. Therefore, nest properties can influence host and parasite reproductive success and the outcome of host-parasite interactions (Heeb *et al.*, 2000). In cavity-nestling birds in particular, the microclimatically stable environment of nests and the presence of an abundant food supply offer excellent breeding conditions for ectoparasites (Collias & Collias, 1984).

Ectoparasites may result in costs for nestlings in terms of compromised growth and condition (Richner *et al.*, 1993; Heeb *et al.*, 1998, 2000; Tomás *et al.*, 2008; Brommer *et al.*, 2011; Cantarero *et al.*, 2013a) because of the direct loss of nutrients and reduced metabolic capacity resulting from parasite feeding activities (Simon *et al.*, 2004). Moreover, in iteroparous species, the effect of parasites on offspring development may, in turn, affect reproductive decisions of parents when

they face the classic trade-off in the investment of limiting resources between current and future reproductive events in order to maximise their own individual fitness (reviewed in Edward & Chapman, 2011 and Alonso-Alvarez & Velando, 2012). In this context, increased reproductive effort to save parasitized offspring might impair parental future reproductive success or survival (e.g. Richner & Tripet, 1999). On the other hand, adults might also suffer direct effects of ectoparasite blood sucking, as in the case of incubating and brooding females, which lead to energetic constraints and loss of condition (Oppliger *et al.*, 1994). Ectoparasites may additionally induce costly immune, inflammatory responses (Møller *et al.*, 2005; Owen *et al.*, 2009) and physiological stress (Martínez-De la Puente *et al.*, 2011).

Only two studies have tested the effect of nest-dwelling ectoparasites on nestling oxidative stress levels (De Coster *et al.*, 2012; Wegmann *et al.*, 2015), manipulating the abundance of a single ectoparasite, the hen flea (*Ceratophyllus gallinae*), in great tit (*Parus major*) nests. Here we study the effect of nest-dwelling ectoparasite loads on the oxidative balance of breeding adults and nestlings in another well known cavity-nesting passerine species, the pied flycatcher (*Ficedula hypoleuca)*. The most common ectoparasites in nests of Iberian pied flycatchers are mites (*Dermanyssus gallinoides)* (more than 60 % prevalence), blowfly larvae (*Protocalliphora azurea)* and fleas (both between 40 % and 50 % prevalence). Strong effects on nestling growth and mortality in our and other populations has been reported for three types of ectoparasites (Merino & Potti, 1995, 1996, 1998; Potti *et al.*, 1999, 2002; Potti, 2007; Lobato *et al.*, 2005, 2008; Moreno *et al.*, 1999, 2008, 2009; Tomás *et al.*, 2007, 2012; Martínez-De la Puente *et al.*, 2009, 2010; Cantarero *et al.*, 2013b). Moreover, we must take into account that nest ectoparasites are a community of species and the decline of one species may allow the proliferation of others (Heeb *et al.*, 2000). This, together with potential multiplicative combined effects of ectoparasites, as in the case of the pied flycatcher, makes it biologically meaningful to study the effects of the entire community of ectoparasites on host physiological parameters related to oxidative stress. Therefore, here we have reduced the abundance of all ectoparasites by a heat treatment of nest boxes. We have previously shown that this procedure significantly reduces ectoparasite abundance and exerts a positive effect on nestling wing and tarsus length (Cantarero *et al.*, 2013a).

In order to properly evaluate the redox balance of individuals, measures of antioxidant capacity and oxidative damage must be obtained simultaneously (Costantini & Verhulst, 2009; Monaghan *et al.*, 2009; Pérez-Rodríguez, 2009). Here we use plasma malondialdehyde (MDA) levels for the assessment of potential oxidative damage accompanying ectoparasite loads. MDA is a by-product of peroxidative decomposition of unsaturated lipids (Halliwell & Gutteridge, 2007). MDA is often considered a presumptive marker of oxidative stress (Mateos *et al.*, 2005; Halliwell & Gutteridge, 2007; Sepp *et al.*, 2012). To monitor antioxidant defences of pied flycatchers, we used two independent markers: total antioxidant status of plasma (TAS) and total glutathione (tGSH) levels in red blood cells. TAS measures the capacity of plasma samples to inhibit a redox reaction induced by free radicals (Miller *et al.*, 1993; Cohen *et al.*, 2007) and is primarily the result of the pooled effect of all extracellular antioxidant compounds of the blood (Costantini, 2011). Glutathione is the predominant low molecular weight tripeptide thiol found in animal cells, functioning in the reduction of the disulphide linkages of proteins and in the protection of cells against free radicals, and is often considered as the most important intracellular antioxidant (Meister, 1991; Wu *et al.*, 2004). In addition, to test whether ectoparasites impaired the nutritional condition of adult females and nestlings, we measured levels of triglycerides (marker of lipid amounts) and uric acid (a marker of protein breakdown; e.g. Alonso-Alvarez & Ferrer, 2001; Hõrak *et al.*, 2006; Costantini *et al.*, 2008). Uric acid and triglycerides in plasma are correlated with TAS and MDA values, so we additionally used these biochemical parameters as covariates for oxidative stress markers (Cohen *et al.*, 2007; Romero-Haro & Alonso-Alvarez, 2014).

Here we examine the effects of a complete natural ectoparasite fauna on oxidative stress of both nestlings and breeding females in cavity-nesting birds. We predicted that:

1. Individuals in control nests would show higher levels of MDA (oxidative damage) than in experimental nests.

2. Ectoparasites would have an effect on antioxidant markers (TAS and tGSH). However, it is difficult to predict the sign of this latter effect, as antioxidants may be exhausted due to their involvement in tissue protection or, rather, through being mobilized to protect against free radicals (Calabrese, 2007;

Costantini & Verhulst, 2009; Costantini *et al.*, 2010). Therefore, we also explored the relationship between oxidative damage and antioxidant markers.

3. Individuals in control nests would show an impaired nutritional state due to the direct effects of ectoparasitism.

METHODS

General field methods

The study was conducted during the spring 2012 in a montane forest of Pyrenean oak (*Quercus pyrenaica*), at 1200 m in Valsaín, central Spain (40˚54′N, 4˚01′W) where long-term studies on cavity-nesting birds have been ongoing since 1991 (see Cantarero *et al.*, 2013a for general description). In the study area there are 552 nest boxes (see Lambrechts *et al.*, 2010 for dimensions, structure and placement of nest boxes) occupied by pied flycatchers, great tits, nuthatches (*Sitta europaea*) and blue tits (*Cyanistes caeruleus*).

We followed breeding activities from the early stages of nest construction to fledging in nest boxes occupied by pied flycatchers. Egg laying in the pied flycatcher population typically begins in late May and the modal clutch size is six. No brooding is observed after nestlings attain 7 days of age (Sanz & Moreno, 1995), and chicks usually fledge at the age of 17 days. Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined.

Shortly before fledging, at day 13 (hatching date = day 1), nestlings were ringed, weighed and measured. Body mass was obtained with a Pesola® spring balance to the nearest 0.25 g and tarsal length was measured with digital callipers (precision 0.01 mm). We took a blood sample of about 120 µl from the brachial vein that was collected in heparinised microcapillaries. Blood samples (N = 268) were stored in Eppendorf tubes in an ice-box until returning to the lab in the same day. Plasma was separated from blood by centrifugation (10 min at 12000 rpm)

and then both fractions were stored at -80 °C until analysed for assaying TAS, MDA, tGSH, uric acid and triglycerides (see below).

Parental individuals were captured in their nest boxes with traps while feeding nestlings of 7-8 days, ringed if necessary or identified by their ring and measured. Due to their higher exposition to ectoparasite infestations, only females (*N* = 52) were blood-sampled in the same way as nestlings to test the effect of treatment on their physiological parameters.

Haemolysis levels in plasma samples were noted by a visual detection of the red colour of plasma, as a consequence of the release of haemoglobin from red blood cells, in a gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree in order to minimize inter-observer variability. Of the 320 blood samples collected, 71 from nestlings and 18 from females were moderately to highly haemolysed.

Because the total volume of some plasma samples was not sufficient to carry out all physiological or biochemical analyses, we established a priority order of assays as follows: MDA, triglycerides, TAS and uric acid levels. This explains why sample sizes for different measures differ.

Experimental reduction of ectoparasites protocol

Of the 91 nest boxes occupied by pied flycatchers we selected 56 whose laying date was between dates 45 and 51 (1 April = day 1) and assigned nests randomly to two groups. Nests in the control group (*N* = 37) maintained their natural ectoparasite loads, while in the experimental group (*N* = 19 nests), we reduced the number of ectoparasites by a heat treatment for 30 s at 750 W using a microwave oven. This treatment ensured that experimental nests did not contain live arthropods when placed in the nest box (Rendell & Verbeek, 1996). To avoid the loss of water during the heat treatment, the nests were placed into a hermetic plastic container. Furthermore, before returning the original nest, the flame from a butane jet torch lighter (Microtorch GT-3000) was passed across the walls of the nest box to kill ectoparasites that might remain there. Only for the time that the original nests were treated (around 30 min) was a fresh substitute nest introduced into the nest box

(these nests had been collected in previous seasons after being abandoned prior to laying and kept frozen at -20 °C until use). To prevent ectoparasite recolonisation of experimental nests a total of three heat treatments were made in the experimental group: 7 days after clutch completion, when nestlings were 2 days old, and when nestlings were 8 days old. Nests in the control group were visited on the same days and handled in a similar way to experimental ones but without applying any treatment. The efficiency of our treatment to eliminate or significantly reduce ectoparasites in heat-treated nests and abundances of ectoparasite species in control nests has been previously reported in Cantarero *et al.* (2013a) for the same set of nests.

Lipid peroxidation assays

Plasma concentrations of total MDA were calculated following Agarwal & Chase (2002) with some modifications made by Mougeot *et al.* (2009). Assays were carried out in 2 ml screw-top microcentrifuge tubes and all chemical solutions were prepared using ultra pure water (Milli- Q Synthesis; Millipore, Watford, UK). For calibration, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) stock solution (10 µM in 40 % ethanol) serially diluted using 40 % ethanol. Butylated hydroxytoluene solution (25 µl; 0.05 % w/v in 95 % ethanol), 200 µl phosphoric acid solution (0.44 M) and 50 µl thiobarbituric acid (TEP) solution (42 mM) were added to 25 µl of plasma samples (1 : 2.5 dilution in water) or standards. Samples were vortex mixed for 5 s and then heated at 100 °C for 1 h in a dry bath incubator to allow the formation of MDA-TBA adducts. The reaction was then stopped by placing samples on ice for 5 min before 125 µl n-butanol was added and tubes were vortex mixed for 1 min. Tubes were then centrifuged at 14000 rpm and 4 °C for 3 min, before the upper (n-butanol) phase was collected and transferred into a high performance liquid chromatography (HPLC) vial for analysis. Samples (10 µl) were injected into an Agilent 1200 HPLC system (Agilent, Santa Clara, CA) fitted with a 5 µm ACE guard column and 5 µm octadecylsilane 100 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) maintained at 37 ºC. The mobile phase was methanol buffer (40 : 60 v/v), the buffer being 50 mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 M potassium hydroxide solution), running isocratically over

3.5 min at a flow rate of 1 ml min^{-1}. Data were collected using a fluorescence detector (G1321A; Agilent) set at 515 nm (excitation) and 553 nm (emission). High repeatability (Lessells & Boag, 1987, here and hereafter) was shown by both a subset of samples assayed in duplicate and TEP standards (R = 0.706, N = 22, p < 0.001 and R = 0.968, N = 6, p < 0.001, respectively).

Total antioxidant status

TAS was assayed following Miller *et al.* (1993) with some modifications made by Cohen *et al.* (2007). Metmyoglobin was generated by mixing equal volumes of 400 µM myoglobin (M0630-250MG; Sigma-Aldrich, St Louis, MO) and 740 µM potassium ferrocyanide, then passing the mixture through a column of Sephadex (G15-120; Sigma-Aldrich). The chromogen, 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid, ABTS) was mixed in PBS to 153 µM. The standard was made by dissolving a water-soluble α-tocopherol derivative, Trolox, in PBS to 1.7 mM. The assay was run in 96-well flat-bottomed clear microplates on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Temperature was maintained at 37 ºC, and readings were taken at 660 nm. Only one 12-well row was used from the plate at a time. Five microlitres of standard (Trolox) or samples was put separately into the wells. Next, 15 µl metmyoglobin and 250 µl ABTS were sequentially added to each well. A multi-channel pipette was used to simultaneously add 50 µl of 300 µM hydrogen peroxide to all the wells, starting the reaction. Kinetic measurements using the spectrophotometer were taken at 10 s intervals; readings were synchronized to the start of the reaction (i.e., injection of hydrogen peroxide) manually using a timer. The reaction runs for around 10 min. Most of samples were assayed in duplicate and showed high repeatability (R = 0.912, N = 209, p < 0.001).

Intracellular tGSH level

tGSH levels in red blood cells were determined according to Galván & Alonso-Alvarez (2008) with some particular modifications. Red blood cell samples were diluted (1 : 20 w/v) and homogenized in a stock buffer (0.01 M phosphate-buffered

saline, PBS, and 0.02 M ethylenediaminetetraacetic acid, EDTA) using a Mini-BeadBeater (Bio- Spec; Bartlesville, OK) and mixed with an equal volume of 10 % trichloroacetic acid. The mixture was vortexed three times during 5 s each bout within a 10 min period. Afterwards, the mixture was centrifuged (3000 *g*, 15 min, 6°C), and the supernatant separated. Three working solutions were made up in a reaction buffer (125 mM sodium phosphate and 6.3 mM EDTA) as follows: (1) 0.3 mM dihydronicotinamide-adenine dinucleotide phosphate, (2) 6 mM 5,5´-dithio-bis(2-nitrobenzoic acid), and (3) 50 U ml^{-1} GSH reductase. Solutions 1 and 2 were mixed at 7 : 1 volume. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek). To 75 µl of sample (supernatant) we added 240 µl of the mixture of solutions 1 and 2. Then, 20 ml of solution 3 was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH from 0.5 to 0.031 mM. Only one 12-well row was used from the plate at a time. A subset of samples assayed in duplicate showed a high repeatability ($R = 0.929$, $N = 44$, $p < 0.001$).

Measurement of uric acid and triglyceride levels

Uric acid is the main form of nitrogen excretion in birds and an indicator of amino acid catabolism. Uric acid is also a powerful antioxidant whose concentration is frequently positively related to TAS values (Cohen *et al.*, 2007; Hõrak *et al.*, 2007; Pérez-Rodríguez *et al.*, 2008), potentially confounding the interpretation of this marker (Cohen *et al.*, 2007; Costantini, 2011). For this reason uric acid-corrected TAS values are recommended over raw TAS levels (Cohen *et al.*, 2007). Triglyceride concentrations reflect the individual´s state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni, 1998). However, triglyceride levels can be related to MDA levels, either because of an effect of diet (MDA is also present in food) or because MDA may also be influenced by the amount of circulating lipids susceptible to oxidation (Pérez-Rodríguez *et al.*, 2015). For this reason, analyses of MDA levels would benefit from entering triglyceride levels as a covariate (Romero-Haro & Alonso-Alvarez, 2014).

Thus, in this study we analysed the effect of our treatment both on raw and on triglycerides-corrected MDA (Pérez-Rodríguez *et al.*, 2015).

The uric acid oxidase/peroxidase method and the glycerol phosphate oxidase/peroxidase method were used for measuring uric acid and triglyceride levels, respectively (kits 11522 and 11529; Biosystems, Barcelona) and using a Synergy HT Multi-Mode Microplate Reader (BioTek). Reagent volumes and further assay details were implemented according to manufacturer instructions. A subset of samples assayed in duplicate showed a high repeatability for both variables (uric acid, $R = 0.99$, $p < 0.001$; triglycerides, $R = 0.94$, $p < 0.001$; $N = 45$ in both cases).

Statistical analyses

Statistical analyses were conducted using STATISTICA (version 7.0; StatSoft). MDA and triglyceride levels of nestlings were normalized through logarithmic transformation. The remaining physiological variables were normally distributed.

Although degree of haemolysis in plasma of nestlings and females was not affected by treatment (Mann-Whitney *U*-test: females, $U = 276.500$, $N = 52$, $p = 0.485$; nestlings, $U = 8142.000$, $N = 266$, $p = 0.837$), haemolysis may affect physiological variables due to a possible efflux of intracellular pro-oxidants and antioxidant molecules in plasma that could alter levels of oxidative markers measured in serum, thereby confounding interpretation of results. Thus, we controlled for this factor in all physiological analyses.

To control for variations in oxidative levels within the control group due to differences in specific ectoparasite abundances, we explored ectoparasite prevalences and possible associations between abundances, as well as correlations between physiological parameters of hosts and ectoparasite abundances within the control group.

Then, we tested the effect of ectoparasite removal on MDA concentration, TAS and tGSH and for triglycerides, uric acid levels and body condition. For female analyses we used treatment as an explanatory factor. In the case of nestling analyses, we included nest identity nested within treatment as a random factor and treatment as a fixed factor. In both cases we controlled for uric acid in TAS

analyses. Effects of treatment on MDA levels were also controlled and non-controlled for variability in triglyceride levels (see above). The models testing the effect of the treatment on body condition included body mass as a dependent variable and tarsus length as a covariate (García-Berthou, 2001).

Cantarero *et al.* (2013a) found negative effects of ectoparasites on tarsus and wing lengths of nestlings, but no correlation between brood size and treatment. However, brood sizes might introduce possible confounding effects on physiological levels. Thus, we performed preliminary tests to control for possible effects of brood size and body condition (tarsus length and body mass) on females and nestling data. Only in cases in which some of these had an effect in physiological variables, were they finally introduced as covariates in initial models (only nestling body mass for triglyceride models and brood size and body mass for female tGSH models).

Finally, we tested covariation between antioxidants and MDA levels by general linear model (GLM) analyses, controlling by haemolysis degree and treatment in all cases and uric acid for TAS analyses. Moreover, we used Student´s *t*-tests to analyse differences between physiological female levels and intra-brood means of nestling levels. Effect sizes were calculated as partial $\eta 2$, i.e. the ratio of variance accounted for by an effect plus its associated error variance within the model.

G*Power (version 3.1.9.2; University of Kiel, Germany) was used to estimate the power to detect large effects ($f = 0.40$) with our samples sizes. Moreover, we calculated observed power and effect sizes for the effect of treatment.

RESULTS

Within the control group, 26 nests (70 %) were parasitized by more than one ectoparasite species at time. Mites had the highest prevalence (36 control nests, 97 %), blowflies were present in 23 of these control nests (62 %) and fleas were found in nine nests (24 %). There were no correlations between abundances of three ectoparasite species (all $p > 0.05$) in control nests. There were no correlations

between mean levels of oxidative parameters and three types of ectoparasite abundances within the control group for both females and nestlings (all $p > 0.05$).

Although female oxidative damage, as measured by MDA levels, was similar for both groups (Table 1; Figure 1.a), females in experimental nests showed significantly higher levels of extracellular antioxidants measured by TAS (Figure 1.b), and higher concentrations of the endogenous antioxidant tGSH in red blood cells (Figure 1.c) than those in control nests. For nestlings, significant differences between treatments were only found for tGSH levels (Table 2), these being higher in treated nests (Figure 1). Results for effects of treatment on MDA levels (Tables 1, 2) did not change when plasma triglyceride levels were entered as a covariate.

For females, there were no correlations between physiological parameters analysed (GLM analyses, all $p > 0.30$). Although MDA and TAS levels of nestlings were not affected by treatment, MDA negatively covaried with TAS as dependent variable (GLM analyses, $F_{1,134} = 5.47$, $p = 0.021$; $\beta = -0.014$) after controlling by uric acid levels, degree of haemolysis, treatment and nest identity. The rest of the covariations between physiological parameters of nestlings were not significant (GLM analyses, all $p > 0.10$).

Triglycerides and uric acid levels of females were not affected by treatment (Table 3). The same was found for nestling uric acid levels (Table 3). Triglycerides of nestlings were not affected by treatment (Table 3), although they showed a positive association with body mass (GLM analysis, $F_{1,239} = 6.49$, $p = 0.011$; $\beta = 0.250$). Body condition, measured as tarsus length-corrected body mass, of females and nestlings was not affected by treatment (Table 3).

Table 1. *Results of GLM analyses of adult female MDA, TAS and tGSH levels (minimal models are selected by backward elimination of non-significant terms to improve models).*

	Num. Df	*F*	*p*	*Power (f =0.4)*	*Obs.effect Size*	*Obs. Power*
MDA						
Full model						
Treatment	1	0.008	0.927	0.669	0.013	0.051
Haemolysis	2	8.122	<0.001*			
Error	*48*					
Minimal model						
Haemolysis	2	8.484	<0.001*			
Error	*49*					
TAS						
Full model						
Treatment	1	3.746	0.064	0.427	0.386	0.403
Uric acid	1	39.213	<0.001*			
Haemolysis	2	1.643	0.214			
Error	*25*					
Minimal model						
Treatment	1	5.543	0.026*	0.429	0.453	0.523
Uric acid	1	37.596	<0.001*			
Error	*27*					
tGSH						
Full model						
Treatment	1	10.299	0.003*	0.616	0.495	0.797
Haemolysis	2	3.015	0.059			
Brood size	1	5.012	0.030*			
Body mass	1	3.369	0.073			
Error	*42*					
Minimal model						
Treatment	1	6.584	0.014*	0.618	0.386	0.587
Haemolysis	2	3.952	0.026*			
Error	*44*					

Statistical power calculated a priori to detect large effects (f = 0.4), observed effect sizes (based on Partial η^2) and observed power are included for the effect of the treatment. * Significant difference (α = 0.05).

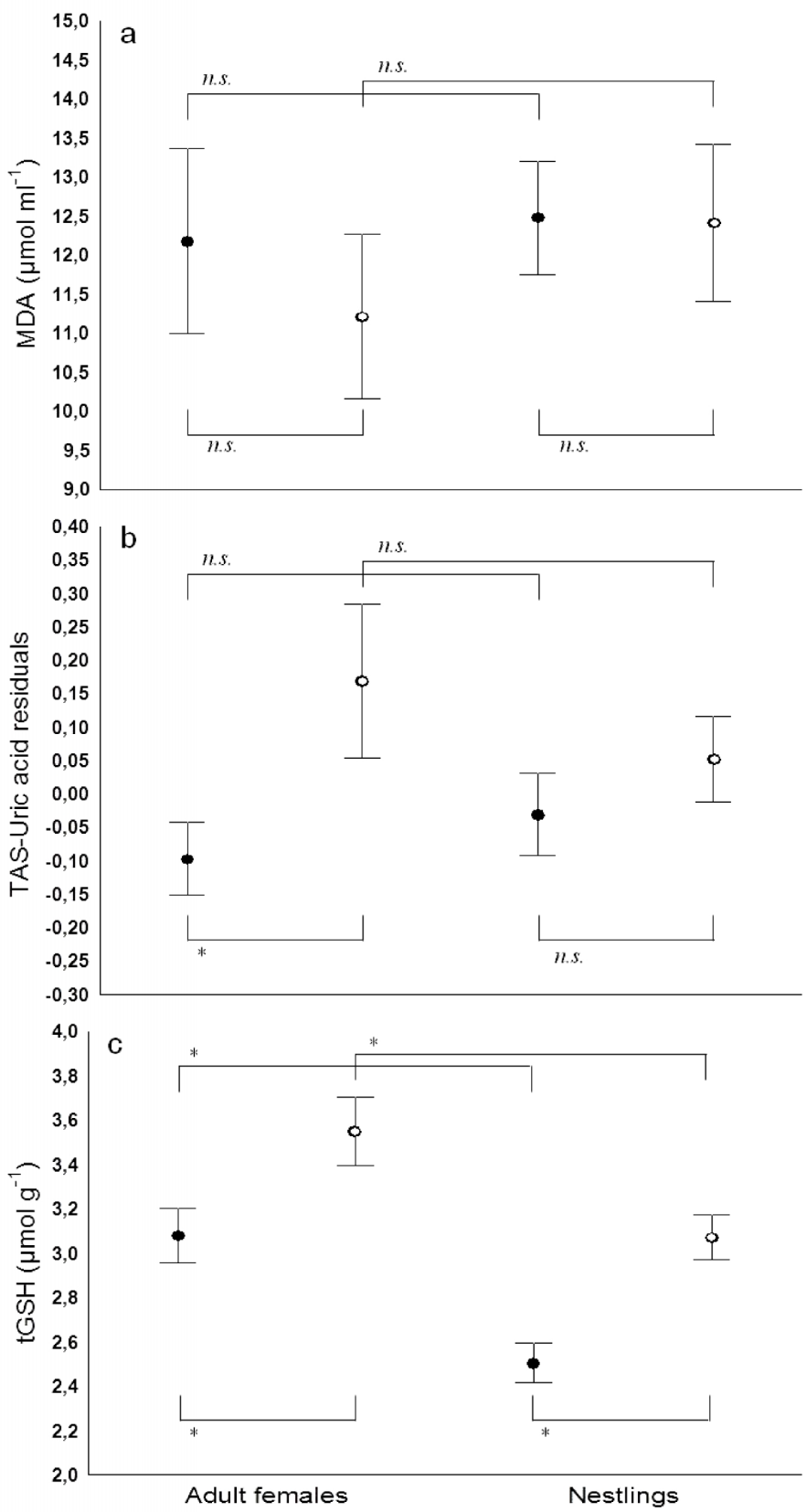

Figure 1. *Effect of experimental removal of nest-dwelling ectoparasites on* ***a)*** *plasma MDA,* ***b)*** *TAS (corrected for uric acid levels), and* ***c)*** *tGSH in erythrocytes. Filled circles: control nests; open circles: heat-treated nests. Values are mean ± SE levels. * indicates significant differences (α = 0.05); n.s. indicates non-significant differences.*

Table 2. *Results of GLM analyses of nestling MDA, TAS and tGSH levels (minimal models are selected by backward elimination of non-significant terms to improve models).*

		df	*F*	*p*	*Power f = 0.4*	*Obs.effect Size*	*Obs. power*
MDA							
Full model							
Treatment	Fix	1, 52.4	0.091	0.764	0.668	0.041	0.057
Haemolysis	Fix	2, 193	39.709	<0.001*			
Nest (Treatment)	Rand	51, 193	3.298	<0.001*			
Error		*193*					
Minimal model							
Haemolysis	Fixed	2, 193	39.709	<0.001*			
Nest (Treatment)	Rand	52, 193	3.235	<0.001*			
Error		*193*					
TAS							
Full model							
Treatment	Fix	1, 51.2	0.085	0.772	0.642	0.095	0.085
Uric acid	Fix	1, 117	210.546	<0.001*			
Haemolysis	Fix	2, 117	1.686	0.189			
Nest (treatment)	Rand	48, 117	6.359	<0.001*			
Error		*117*					
Minimal model							
Uric acid	Fix	1, 119	205.219	<0.001*			
Nest (Treatment)	Rand	49, 119	6.139	<0.001*			
Error		*119*					
tGSH							
Full model							
Treatment	Fix	1, 50.9	13.854	<0.001*	0.668	0.522	0.878
Haemolysis	Fix	2, 211	8.503	<0.001*			
Nest (Treatment)	Rand	51, 211	3.809	<0.001*			
Error		*211*					

Statistical power calculated a priori to detect large effects (f = 0.40), observed effect sizes (based on Partial η^2) and observed power are included for the effect of the treatment. * Significant difference (α = 0.05)

Independently of treatment, levels of triglycerides and uric acid were lower in females than in nestlings (Table 3; *t*-test triglycerides, t_{92} = -7.111, p < 0.001; uric acid, t_{78} = -2.997, p = 0.004). By contrast, tGSH levels (Figure 1.c) were higher in females than in nestlings (*t*-test tGSH, t_{101} = 4.213, p < 0.001), while there were no differences for TAS (Figure 1.b) and MDA (Figure 1.a) (*t*-test, all p > 0.500).

Table 3. Mean ± SE (sample size in parentheses) of female and nestling indicators of nutritional condition in relation to treatment.

	Control	*Heat-treated*	*F*	*p*	*Power f = 0.4*	*Obs. Eff.size*	*Obs. Power*
Adult females							
Triglycerides	138.03±4.69	128.14±5.86	1.74	0.195	0.591	0.211	0.211
Uric acid	10.42±0.35	10.17±0.46	0.59	0.447	0.431	0.146	0.100
Body mass[a]	12.71±0.214	12.45±0.20	1.20	0.279	0.669	0.153	0.151
Nestlings							
Triglycerides	227.32±3.94	197.63±4.96	2.46	0.125	0.669	0.217	0.256
Uric acid	12.18±0.26	12.59±0.29	1.89	0.176	0.645	0.204	0.222
Body mass[a]	14.05±0.05	14.05±0.07	0.00	0.982	0.669	0.022	0.052

Statistical power calculated a priori to detect large effects (f = 0.40), observed effect sizes (based on Partial η^2) and observed power are included for the effect of the treatment. [a] Tarsus length corrected; LS Means

DISCUSSION

Our results support the hypothesis that ectoparasites impose a physiological cost for cavity-nesting birds. Although there was a lack of significant differences in MDA levels between the two treatments (prediction 1), our prediction about the negative effects of ectoparasites on host antioxidants (2) was supported by the lower tGSH levels in both females and nestlings exposed to ectoparasites and by the lower TAS values of females in control nests. These results indicate that ectoparasites exposed cavity-nesting birds to an oxidative challenge that depleted antioxidant defences. Moreover, exploring associations between oxidative damage and antioxidants, we found a negative relation between antioxidant status and MDA levels in nestlings, but not in females. Finally, and contrary to our prediction (3), those biochemical parameters related to nutritional state (triglycerides and uric acid levels) were not affected by treatment.

Cellular GSH levels reflect a steady-state balance between synthesis and loss. At normal levels of oxidative stress there is essentially no net loss of GSH through oxidation (Griffith, 1999). However, if pro-oxidant levels increase sufficiently, GSH protects cells, reacting rapidly with peroxides and producing (glutathione disulphide, GSSG, an oxidized form of GSH). Although the enzyme GSH reductase reduces GSSG back to GSH, GSSG is

rather degraded extracellularly and GSH is not taken up intact by cells (Griffith, 1999; Wu *et al.*, 2004). The depletion of cellular tGSH levels in parasitized individuals may indicate an increase in RONS produced during inflammatory responses to ectoparasite bites (Baron & Weintraub, 1987; Owen *et al.*, 2010) or as a consequence of an increase in metabolic activity (Finkel & Holbrook, 2000). Accordingly, Cantarero *et al.* (2013a) found that ectoparasites increase begging intensity in nestlings, and they detected a positive correlation between begging intensity and provisioning rates of adults. This presumably raised energetic costs and metabolic activity in both cases, potentially leading to an oxidative challenge (Moreno-Rueda *et al.*, 2012).

Another piece of evidence about the possible costs of ectoparasitism on hosts is a decrease in plasma antioxidant status, a measure that assesses the levels of nonenzymatic antioxidants, such as vitamins A, C and E, and carotenoids. All of these are progressively used up to protect the cell membrane and prevent lysis (Lesgards *et al.*, 2002; Bertrand *et al.*, 2006). Low molecular weight and lipid-soluble antioxidants like tocopherol (vitamin E), have been linked mainly to lipid peroxidation, while the water-soluble ascorbic acid (vitamin C) has been suggested to be part of a first line of defence (Dotan *et al.*, 2004) and can also protect membranes against peroxidation by enhancing the activity of tocopherol (Sies & Stahl, 1995). In this line of reasoning, the negative correlation between MDA and TAS in nestling values supports the role of TAS in protection against lipid peroxidation. Our results showed that adult females, but not nestlings, had less antioxidant capacity in parasitized than heat-treated nests. The fact that only females showed this effect would suggest a stronger challenge imposed by ectoparasites for the parents compared to nestlings. This may be a consequence of the intense flight activity during nestling feeding. In fact, it has been recently shown in birds that flight effort may contribute to the depletion of the antioxidant defences in order to maintain redox homeostasis (Costantini *et al.*, 2008). In contrast, nestlings experience very low activity levels in nests and when measured have been shown to have passed the peak of tissue growth and cell proliferation (Lundberg & Alatalo, 1992), which may also be sources of oxidative stress (Metcalfe & Alonso-Alvarez, 2010 and references therein). These results are in accordance with studies in which parasite-mediated depletion of antioxidant defences have been reported (Hõrak *et al.*, 2004a; Mougeot *et al.*, 2009; Van de Crommenacker *et al.*, 2012). However, there are some studies that report opposite

results or a lack of effects (Hõrak *et al.*, 2004b; Pap *et al.*, 2011; De Coster *et al.*, 2012; Wegmann *et al.*, 2015), which can be explained by hormetic compensation of the antioxidant machinery (Hõrak *et al.*, 2007), or due to differential adjustments in maternal investment that can affect self-maintenance and offspring physiological status (De Coster *et al.*, 2012; Wegmann *et al.*, 2015).

In birds a decline in erythrocyte tGSH levels was also detected under exposure to experimentally high levels of RONS, though no effect in TAS values was found (Galván & Alonso-Alvarez, 2009; Alonso-Alvarez & Galván, 2011). Although we have not found effects of ectoparasites on TAS in nestlings, Cantarero *et al.* (2013a) showed that the same nestlings exposed to ectoparasites reduced their growth compared with nestlings from the treated group, which suggests that the resources invested by cells in maintaining oxidative stress under the control may have affected cell proliferation and thereby tissue growth. Furthermore, the decrease in nestling tGSH in control nests is consistent with an incomplete compensation of antioxidant defences. However, we cannot discount that our manipulation could have had some effect on nestling morphological traits used as signals in parent-offspring communication, such as the carotenoid-based colour of the nestling mouth (e.g. De Ayala *et al.*, 2007; Dugas, 2012). These carotenoid-based traits can be particularly susceptible to alterations in the oxidative status of the individual (Von Schantz *et al.*, 1999; Pérez-Rodríguez, 2009), which could have influenced sibling and parent behaviour during nestling and post-fledging periods. Unfortunately, we have not assessed these ornamental traits in our birds. However, we have previously shown a lack of effect of the ectoparasite abundance treatment on provisioning rates to nestlings during development (Cantarero *et al.*, 2013a).

Although we have not found effects of ectoparasites on MDA, this may be due to adjustments in the antioxidant system (tGSH and TAS) induced by ectoparasites. Similar patterns have been observed in coccidian infection in mammals (Rakhshandehroo *et al.*, 2013), studies of which report a depletion in antioxidant defences to maintain a safe level of oxidative damage after a peak of infection. Our results may therefore suggest that individuals have the ability to cope, at least to some degree, with any increase in oxidative stress due to parasitism. However, the lack of effects of the treatment on some biomarkers may also be due to our limited sample size. A priori G*Power tests were

performed to estimate the minimum sample size required to detect effects of our treatment on physiological variables. Minimum sample sizes required to detect (α = 0.05; 1-β = 0.80) large (f = 0.40), medium (f = 0.25) and small (f = 0.10) effects of our treatment on physiological variables were 52, 128 and 787 nests, respectively. Accordingly, Cohen´s conventions (1992) proposes at least 26 cases per group to test differences between two treatments in order to detect, at least, large size effects at p < 0.05. Here, however, we had a maximum of 19 nests in the manipulated group, which a priori implies intermediate power levels to detect large effects (range, 0.43-0.67; Tables 1, 2, 3). We also estimated a low observed power for both adult female and nestling analyses, only reaching the 0.80 threshold in the case of tGSH (Table 2). The variable sensitivity to the effect of treatment may be due to the large differences of variance among response variables. Therefore, an interpretation of these null results might be highly speculative.

Triglyceride concentrations reflect the individual´s state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni, 1998) and are positively related to body mass, as reported in the Results. Uric acid levels are also good indicators of nutritional state due to active protein catabolism during the last phase of fasting (Alonso-Alvarez & Ferrer, 2001; McCue, 2010). Thus, these blood metabolites may reflect variation in the basic nutritional state (Hõrak *et al.*, 2006), and the lack of effects of our experiment on these may be related to the lack of differences in body condition as shown in the Results. This could be explained by conditions during the year of study (2012), which were especially favourable, as nestlings attained their largest masses since the inception of the long-term study (Cantarero *et al.*, 2013a).

Interestingly, lower levels of triglycerides of adult females when compared with nestlings may reflect costs of flight, causing tissues to increased hydrolysis of triglycerides from adipose free fatty acids and glycerol and oxidation of free fatty acids by muscle activity (Schwilch *et al.*, 1996). Additionally, high uric acid levels reported for nestlings, when compared with females, may be a consequence of nestling diet due to plasma uric acid levels in birds increasing a few hours after feeding (Romero-Haro & Alonso-Alvarez, 2014 and references therein). On the other hand, similar to our findings, Isaksson *et al.* (2005) reported higher levels of tGSH in adult females than in great tit

nestlings. They explained this as an adaptive response (i.e. hormesis; Calabrese, 2007; Costantini *et al.*, 2010) to the physical strain and other stressors experienced by breeding adults, as compared to nestlings sitting mostly immobile, warm, and safe in the nest box along with a not fully developed defence system (Isaksson *et al.*, 2005). In females, tGSH levels are negatively associated with dependent brood size and positively correlated with their body mass. This might indicate that tGSH in females is related to effort-dependent condition, which in turn is affected by parental metabolic exertion.

To summarize, we have reported here negative effects of nest ectoparasites on nestling physiological traits related to oxidative stress. These may contribute to explain the detrimental effects on nestling development and fledging success demonstrated for the same set of nests (Cantarero *et al.*, 2013a). We also show that breeding females are also affected physiologically by nest-dwelling ectoparasites, as shown in other studies with respect to other health parameters (e.g. Tomás *et al.*, 2007). Here we demonstrated that ectoparasites may impose an oxidative challenge to cavity nesting birds: breeding females and nestlings from parasitized nests managed to maintain oxidative damage levels, but at the cost of an impaired antioxidant system. Though the potential long-term effects of our findings are still being studied, these parasite-induced oxidative challenges can lead to reduced survival or resource allocation to future reproduction (Richner & Tripet, 1999; Fitze *et al.*, 2004a, 2004b). A large number of studies have shown the links between oxidative stress and antioxidant defences with fitness traits including fecundity, egg-laying capacity and short- and long-term viability (Blount *et al.*, 2004; reviews in Monaghan *et al.*, 2009; reviews in Dowling & Simmons, 2009; Helfenstein *et al.*, 2010; Metcalfe & Alonso-Alvarez, 2010; Saino *et al.*, 2011). Moreover, associations between recruitment probability and pre-fledging oxidative damage (Noguera *et al.*, 2011) and between antioxidant defences and winter survival (Norte *et al.*, 2008) have been reported in wild birds. Here, we acknowledge that the use of microwaves to reduce ectoparasite loads may kill microorganisms present in nests, both beneficial and pathogenic (Goodenough & Stallwood, 2010; González-Braojos *et al.*, 2012), and that we therefore cannot exclude possible interactions of their presence/absence on our results. However, contrary to the idea that nest-dwelling ectoparasites are of minor importance for breeding success and survival (e.g. Eeva *et al.*, 1994; Bauchau, 1997), we here report detectable costs of ectoparasitism not only on behavioural and developmental traits (Cantarero *et*

al., 2013a), but also on key physiological parameters, which may compromise host fitness.

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EL ESTRÉS OXIDATIVO DURANTE EL DESARROLLO TEMPRANO: ASOCIACIONES CON EL SEXO, LAS CONDICIONES DE CRÍA Y LOS RASGOS FISIOLÓGICOS PARENTALES EN POLLUELOS

López-Arrabé, J., Cantarero, A., Pérez-Rodríguez, L., Palma, A. & Moreno, J. 2016. Oxidative stress in early life: associations with sex, rearing conditions, and parental physiological traits in nestling pied flycatchers. *Physiological & Biochemical Zoology*, 89:83-92

RESUMEN

Las condiciones experimentadas durante el desarrollo juvenil afectan a la eficacia biológica de los organismos. Durante el desarrollo temprano, los niveles de estrés oxidativo pueden ser particularmente altos como resultado del elevado metabolismo y un sistema antioxidante relativamente inmaduro, lo que puede tener consecuencia a medio y largo plazo sobre la eficacia biológica. Aquí exploramos la variación en los niveles de estrés oxidativo medido durante el desarrollo temprano en relación con el sexo, las condiciones de cría (fecha de eclosión y tamaño de nidada), y la condición y estado oxidativo parental en una población silvestre de Papamoscas cerrojillo (*Ficedula hypoleuca*) durante dos años de estudio. Un marcador de la capacidad antioxidante total (TAS) en plasma y niveles totales de glutatión (tGSH) en glóbulos rojos, así como un marcador de daño oxidativo en lípidos plasmáticos (malondialdehído, MDA) fueron medidos simultáneamente. Nuestros resultados muestran que los niveles de tGSH se asociaron con la actividad oxidativa parental, con una correlación negativa con el MDA materno y positiva con los niveles de tGSH de ambos progenitores, indicando una alta heredabilidad estimada. Esto sugiere que la fisiología y los genes parentales podrían ser determinantes para la composición de los antioxidantes endógenos de su descendencia. Además, se encontró que los niveles de tGSH fueron más altos en hembras que en machos y que la fecha de eclosión se asoció positivamente con las defensas antioxidantes (mayores TAS y niveles de tGSH). Estos resultados sugieren que diferentes componentes del equilibrio redox están relacionados con varios factores ambientales e intrínsecos, incluyendo factores de influencia parental. Los estudios experimentales futuros deben profundizar en el estudio de la contribución de cada uno de ellos sobre el estado oxidativo de polluelos y en cómo el estrés oxidativo experimentado en fases tempranas afecta a la formación del fenotipo adulto y a su eficacia biológica.

ABSTRACT

Conditions experienced during juvenile development can affect the fitness of an organism. During early life, oxidative stress levels can be particularly high as a result of the increased metabolism and the relatively immature antioxidant system of the individual, and this may have medium- and long-term fitness consequences. Here we explore variation in levels of oxidative stress measured during early life in relation to sex, rearing conditions (hatching date and brood size), and parental condition and levels of oxidative markers in a wild population of the pied flycatcher (*Ficedula hypoleuca*) followed for 2 yr. A marker of total antioxidant status (TAS) in plasma and total levels of glutathione (tGSH) in red blood cells, as well as a marker of oxidative damage in plasma lipids (malondialdehyde, MDA), were assessed simultaneously. Our results show that nestling tGSH levels were associated with parental oxidative status, correlating negatively with maternal MDA and positively with total GSH levels of both parents, with a high estimated heritability. This suggests that parental physiology and genes could be determinants for endogenous components of the antioxidant system of the offspring. Moreover, we found that tGSH levels were higher in female than in male nestlings and that hatching date was positively associated with antioxidant defenses (higher TAS and tGSH levels). These results suggest that different components of oxidative balance are related to a variety of environmental and intrinsic -including parental- influencing factors. Future experimental studies must disentangle the relative contribution of each of these on nestling oxidative status and how the resulting oxidative stress at early phases shape adult phenotype and fitness.

Keywords: environmental conditions, total glutathione (GSH), heritability, malondialdehyde (MDA), total antioxidant status (TAS), early-life effects.

INTRODUCTION

Oxidative stress -the imbalance between the rate of production of reactive oxygen and nitrogen species and the antioxidant machinery- may affect several fitness-related traits, shaping animal life-history evolution (Halliwell & Gutteridge, 2007; Costantini, 2008; Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Costantini *et al.*, 2010; Metcalfe & Alonso-Alvarez, 2010). Juvenile development is a life stage in which oxidative stress levels can be particularly high (Gaál *et al.*, 1996; Nussey *et al.*, 2009), as a result of the high rate of metabolism required for growth, which an immature antioxidant system has difficultly matching (Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010 and references therein). Such early oxidative stress during early development may have long-lasting fitness consequences later in life (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2006, 2007; Norte *et al.*, 2009; Koivula *et al.*, 2011; Noguera *et al.*, 2011; reviews in Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Costantini, 2014). Therefore, identifying the main factors determining oxidative status during early development is essential to obtain an integral understanding of life-history trade-offs.

The level of oxidative stress experienced by an organism can be influenced by a combination of genetic and environmental factors. Evidence of a genetic contribution to the generation of reactive oxidative metabolites and resistance to oxidative stress has been provided for a few model species (Costantini & Dell´Omo, 2006; Kim *et al.*, 2010; Losdat *et al.*, 2014). The comparison between related individuals (i.e., parents and offspring) allows inferring of the relative contribution of genes to the variability of a specific trait (Falconer & Mackay, 1996). This provides an estimate of the narrow sense heritability (h^2), which is defined as the ratio of the additive genetic variance to the phenotypic variance (Lynch & Walsh, 1998).

Several studies also support the existence of a contribution of environment to the level of oxidative stress experienced by individuals during early life stages (e.g., Costantini *et al.*, 2006; Rubolini *et al.*, 2006; Monaghan, 2008; Bourgeon *et al.*, 2011; Lushchack, 2011; Stier *et al.*, 2014). For altricial birds, nest related conditions determine an immediate environment that influences nestling oxidative stress (e.g., Costantini & Dell´Omo, 2006; Costantini *et al.*, 2006; Losdat *et al.*, 2014; López-Arrabé *et al.*, 2015).

The number of siblings in the nest, for instance, affects the amount of food received by nestlings, their growth rate, and the energy spent on competition. This could lead to a higher metabolism rate with an overproduction of free radicals and a differential antioxidant intake and allocation, which could ultimately influence nestling oxidative stress (Costantini *et al.*, 2006; Alonso-Alvarez *et al.*, 2007; but see Losdat *et al.*, 2010). In turn, the quantity/quality of the food provided by parents ultimately depends on the productivity of the environment, which is well known to vary seasonally (Martin, 1987) and can alter antioxidant status either directly (Blount *et al.,* 2003; Costantini 2010) or by modifying growth trajectories (Alonso-Alvarez *et al.*, 2006, 2007). Thus, breeding date is also a critical factor determining offspring fitness, particularly so in migratory species that face opposed environmental constraints between arriving and breeding too early, when weather conditions are still inadequate, and breeding too late, when odds of success decrease and food availability declines (Brown & Brown, 2000). However, the effects of these two aspects of rearing conditions on nestling oxidative stress remains understudied.

Here we explore sources of variation in oxidative stress experienced by nestlings relative to the body condition and amount of oxidative stress in parents, nestling sex, and rearing conditions (brood size, hatching date) in a wild population of the migratory pied flycatcher (*Ficedula hypoleuca*). Some recent studies have explored the sources of variation of oxidative stress in adult pied flycatchers (e.g., Morales *et al.*, 2008, 2011, 2013b, 2013c; López-Arrabé *et al.*, 2014b). However, although there is evidence of associations between nestling oxidative stress and some environmental factors, such as pollutants (Berglund *et al.*, 2007; Rainio *et al.*, 2013; López-Arrabé *et al.*, 2014a) or the presence of nest-dwelling ectoparasites (López- Arrabé *et al.*, 2015), no comprehensive study exploring patterns in oxidative markers of nestlings has been conducted in this species.

In order to properly evaluate the redox balance of individuals, measures of antioxidant capacity and oxidative damage must be obtained simultaneously (Costantini & Verhulst, 2009; Monaghan *et al.*, 2009; Pérez-Rodríguez, 2009). Here we use plasma malondialdehyde (MDA) levels, a by-product of lipid peroxidation (Halliwell & Gutteridge, 2007), as a measure of oxidative damage (Mateos *et al.*, 2005; Halliwell & Gutteridge, 2007; Sepp *et al.*, 2012). To monitor antioxidant defenses, we use two independent

markers: total antioxidant status (TAS) of plasma and total glutathione (tGSH) levels in red blood cells (RBCs). TAS measures the capacity of plasma samples to inhibit a redox reaction induced by free radicals (Miller *et al.*, 1993; Cohen *et al.*, 2007) and is primarily the result of the pooled effect of all extracellular antioxidant compounds of the blood (Costantini, 2011). Glutathione is a tripeptide thiol functioning in the protection of cells against free radicals, often considered as one of the most important intracellular antioxidants (Meister, 1991; Wu *et al.*, 2004). Both TAS and MDA levels can be affected by the concentration of uric acid and triglycerides, respectively, which may confound the interpretation of these oxidative stress biomarkers (Cohen *et al.*, 2007; Costantini, 2011; Pérez-Rodríguez *et al.*, 2015). Uric acid is the main form of nitrogen excretion in birds and an indicator of amino acid catabolism, but it is also a powerful antioxidant frequently positively related to TAS values (Cohen *et al.*, 2007; Hõrak *et al.*, 2007; Pérez-Rodríguez *et al.*, 2008a). On the other hand, triglyceride levels can be related to MDA levels, either because of an effect of diet (MDA is also present in food) or because MDA may also be influenced by the amount of circulating lipids susceptible to oxidation (Pérez- Rodríguez *et al.*, 2015). Therefore, we also quantified these two plasma metabolites in order to statistically control for their effects in the analyses.

Our main goal was to examine factors affecting oxidative status in nestlings shortly before fledging, addressing the potential contribution of genetic, parental, and environmental effects. We explored oxidative damage and antioxidants in relation to (1) maternal and paternal oxidative status and body condition, (2) sex of nestlings, and (3) hatching date and brood size. We found that levels of the cellular antioxidant GSH were related to parental oxidative status -showing a significant heritability- but were also influenced by hatching date. We discuss the importance of these links in the context of life history, focusing on the potential impact of oxidative stress during nestling development on individual fitness.

METHODS

General Field Methods

The study was conducted during spring of 2012 and 2013 in a montane forest of Pyrenean oak (*Quercus pyrenaica*) at 1200 m above sea level in Valsaín, central Spain (40º54´N, 04º01´W). In the general study area, there are 570 nest boxes (for dimensions, structure, and placement of nest-boxes, see appendix in Lambrechts *et al.*, 2010), around 100 of which are yearly occupied by pied flycatchers.

We followed breeding activities from nest construction to fledging in nest boxes occupied by pied flycatchers. Egg laying in our population of pied flycatchers typically began in late May. Females laid on average six eggs, and chicks usually fledged at the age of 17 d.

We captured adult males and females in their nest boxes while they were provisioning 7-8-days old nestlings, ringed them if necessary, blood sampled, weighed and measured them. Body mass was obtained with a digital scale to the nearest 0.1 g, and tarsal length was measured with a digital calliper (precision 0.01 mm). We took a blood sample of about 120 ml from the brachial vein that was collected in heparinized microcapillaries. We collected blood from adult females during 2012 and 2013 (N = 34) but males only in 2013 (N = 31). Blood samples were stored in Eppendorf tubes in an icebox until returning to the lab on the same day. In the lab, plasma samples were centrifuged (10 min at 12000 rpm) to separate blood cells from plasma, and then both fractions were stored at -80 ºC. If haemolysis occurs during sampling, a possible efflux of intracellular pro-oxidants and antioxidant molecules into plasma could alter levels of oxidative markers, thereby confounding interpretation of results. Thus, haemolysis levels in plasma samples were noted by a visual inspection of red colour of plasma samples. We scored samples from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person scored the degree of haemolysis in order to minimize inter-observer variability.

For both years, on day 13 (hatching date = day 1), nestlings were ringed, weighed and measured, and a blood sample was collected following the same protocol as in

adults. In 2012, we collected blood from all chicks in the nest (N = 199), but in 2013, we took blood samples from two randomly selected nestlings of each nest (N = 74).

Lipid Peroxidation Assay

Plasma concentrations of MDA were analyzed as described by López-Arrabé *et al.* (2014b). Briefly, a standard curve was prepared for calibration, using a 1,1,3,3-tetraethoxypropane stock solution serially diluted in 40% ethanol. Butylated hydroxytoluene, phosphoric acid, and thiobarbituric acid (TBA) solutions were added to each plasma sample and standard. Then, samples were incubated on a dry bath to allow formation of MDA-TBA adducts. After that, pure *n*-butanol was added to each sample and standard. Tubes were vortexed and centrifuged, and the upper phase was collected and transferred into a high-performance liquid chromatography (HPLC) vial for analysis. Samples were injected into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA). Data were collected using a fluorescence detector (G1321A, Agilent Technologies). Repeatability (following Lessells & Boag, 1987), calculated on a set of samples assayed in duplicate, was high (r = 0.722, N = 66, p < 0.001). Interassay coefficient of variability (CV) was 8.08%.

Total Antioxidant Status (TAS)

TAS was analyzed as described by López-Arrabé *et al.* (2014b). As standard for the assays, we used Trolox (a water-soluble α-tocopherol derivative), and TAS levels are expressed in Trolox-equivalent units. The assays were run on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). In order to accurately control the reaction time, only one column of the plate was used at a time. To standard or samples were sequentially added metmyoglobin -a mixed of equal volumes of myoglobin (M0630-250MG, Sigma-Aldrich, St. Louis) and potassium ferricyanate-, ABTS (the chromogen, 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)), and H_2O_2, starting the reaction. Kinetic measurements were immediately started, recording absorbance at 660 nm every 5 s. The temperature was maintained at 37 ºC during assays. All samples were assayed

in duplicate, and results showed a high repeatability (r = 0.981, N = 234, p < 0.001) and an interassay CV of 3.63%.

Intracellular tGSH Level

tGSH levels in RBCs were determined as described by López-Arrabé *et al.* (2014a) and Romero-Haro & Alonso-Alvarez (2015). Briefly, RBC samples were diluted and homogenized in a stock buffer and mixed with trichloroacetic acid. The mixture was vortexed and centrifuged, and the supernatant was separated. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments). To samples (supernatant) we added a mixture of nicotinamide adenine dinucleotide phosphate and 5,50-dithiobis(2-nitrobenzoic acid). Afterward, a GSH reductase solution was added after 15 s, and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH. The assays were performed at 37 ºC, and only one column of the plate was used at a time in order to control reaction times accurately. A set of samples assayed in duplicate showed a high repeatability (r = 0.983, N = 106, p < 0.001) and an interassay CV of 3.89%.

Measurement of Uric Acid and Triglyceride Levels

Following previous studies in birds (e.g., Pérez-Rodríguez *et al.*, 2008a, 2015; Romero-Haro & Alonso-Alvarez, 2015), plasma levels of uric acid and triglycerides were measured using commercial kits (11522 and 11529 from Biosystems, Barcelona) based on the uricase/peroxidase method (Fossati *et al.*, 1980) and the glycerol phosphate oxidase/peroxidase method (Bucolo *et al.*, 1973), respectively. Analyses were run in 96-well plates using the same microplate reader mentioned before. For uric acid, 150 ml of the chromogen were added to 5 ml of each plasma sample or the standard (a 6 mg/dl uric acid solution). For triglycerides, 250 ml of the chromogen were added to 5 ml of each plasma sample or the standard (a 200 mg/dl glycerol solution). In both cases, plates were incubated for 5 min at 37 ºC, subsequently measuring absorbance at 520 nm (for

uric acid) or 500 nm (for triglycerides). A subset of samples assayed in duplicate showed a high repeatability for both variables (uric acid: $r = 0.99$, $p < 0.001$; triglycerides: $r = 0.94$, $p < 0.001$; $N = 45$ in both cases). Interassay CVs were 2.79% and 3.76% for uric acid and triglycerides, respectively.

Sex Determination

The sex of nestlings was determined by the amplification of the CHD sequence present in both W and Z avian chromosomes, by polymerase chain reactions (PCRs) using the primers P2 (50-TCT GCA TCG CTA AAT CCT TT-30) and P8 (50-CTC CCA AGG ATG AGR AAY TG-30; Griffiths *et al.*, 1998). We performed assays on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Reaction conditions were as follows: an initial denaturing step at 94 ºC for 4 min and 30 s was followed by 45 cycles of 94 ºC for 30 s, 49 ºC for 45 s, and 72 ºC for 45 s. A final run of 72 ºC for 5 min completed the program. PCR products were separated by electrophoresis in cyan/yellow loading buffer (Invitrogen, Life Technologies, Carlsbad, CA) at 100 V for 20 min. The amplification products corresponded to CDHW and CDHZ genes. Males are identified by having only the CDHZ band, while females present both CDHZ and CDHW bands.

Statistical Analyses

In order to statistically control for potentially confounding effects, we explored the effect of the degree of haemolysis on the oxidative stress parameters of both nestlings and adults. For nestlings and adult males, haemolysis affected only MDA levels (both $p < 0.001$). For adult females, haemolysis affected MDA ($p < 0.001$) and tGSH ($p = 0.032$) levels. In these cases, we controlled the variables -when entered either as dependent variables or as covariates- by the degree of haemolysis. Also, we ran models restricted to non-haemolysed samples in order to assess the robustness of our results. Uric acid affects plasma TAS measures (Cohen *et al.*, 2007; Pérez-Rodríguez *et al.*, 2008b), so this marker of antioxidant capacity was always controlled for uric acid levels when entered in the models. Also, as recently proposed (Pérez-Rodríguez *et al.*, 2015), statistical models for MDA were run including and excluding triglyceride level as a covariate.

To analyze the relationships between nestling oxidative stress parameters and parental oxidative status and body condition, we ran separate models including nestling MDA, TAS, or tGSH as dependent variables and either female or male parent oxidative stress measures and body condition (residuals of the regression of body mass on tarsus length) as covariates. In all cases, we controlled for hatching date and brood size by including them as covariates. In models analyzing associations with the oxidative status and body condition of the female parent, we also included year of sampling as fixed factor. For data from 2013, we estimated heritability (h^2) through midparent-offspring correlations (Falconer & Mackay, 1996). The slope of the regression of mean offspring measurements on the mean of their parent´s measurements (the midparent value) constitutes a crude measure of heritability when full pedigrees are not available. We have assumed that paternity has been accurately estimated through paternal care at the nest, an assumption that could introduce some error if extra-pair paternity was high in 2013 (for estimates of extra-pair paternity in the population, see Moreno *et al.*, 2013a, 2015).

Also, we analyzed the relationships between nestling oxidative stress parameters and breeding variables (hatching date and brood size) by running models that included nestling MDA, TAS, or tGSH as dependent variables; hatching date, brood size, and nestling body condition as covariates; and sex of nestlings and year of sampling as fixed factors.

Finally, to test the potential relationship between nestling body condition and breeding variables, we ran a model including body mass as a dependent variable; hatching date, brood size, and tarsus length as covariates; and year as a fixed factor.

In all cases, we used mixed models using PROC MIXED in SAS (ver. 9.3) and restricted maximum likelihood to analyze the variation in nestling oxidative variables. Degrees of freedom (*df*) were estimated using the Satterthwaite approximation, thus avoiding possible pseudoreplication. Only nestling MDA levels were log transformed to obtain a normal distribution. The rest of dependent variables were normally distributed. Nest was included as a random factor to avoid pseudoreplication, and individual nestling was used as a sample unit. Moreover, in those cases (only three) in which adult females were captured in the 2 yr of the study, data from one of the years randomly were eliminated to avoid pseudoreplication. To obtain minimal adequate models, we

elaborated full saturated models including in each case the factors mentioned above, and we sequentially removed the less significant terms following a standard backward stepwise procedure until only significant variables were retained.

RESULTS

We found a negative association between tGSH levels in nestlings and adult female MDA (Table 1; Figure 1). Moreover, tGSH levels in nestlings and adult females were significantly and positively correlated (Table 1). A similar positive association was found between nestling and male tGSH levels ($F_{1, 27.6} = 7.35$, $p = 0.011$; *estimate* = 0.440 ± 0.162). Heritability of tGSH levels varied from 0.60 to 0.71 (Figure 2). Although they were separated by less than 2SD from the mean of the sample, two potential outliers might influence results (Figure 2). However, when we repeated the analyses excluding them, the results remained the same ($F1, 24 = 15.74$, $p < 0.001$; $y = 0.3628 + 0.7071x$).

Nestlings that hatched later in the breeding season showed higher levels of tGSH (Table 2) and higher TAS values (Table 1). Moreover, tGSH levels were significantly higher in female than in male nestlings (Table 2; Figure 3). Finally, there were no associations of nestling body condition with brood size or hatching date (both $p > 0.06$).

None of the above mentioned results for MDA levels (Tables 1, 2) changed when plasma triglyceride levels were entered as a covariate in the respective models. In the same way, results were qualitatively similar when we restricted our analyses to nonhemolyzed samples.

Table 1: *Results of mixed models exploring associations between physiological variables of nestling and female pied flycatchers.*

	Estimate ± SE	*Df*	*F*	*P*
Nestling MDA				
Full model				
Hatching date	0.006 ± 0.014	1, 37.0	0.19	0.665
Brood size	-0.044 ± 0.049	1, 44.8	0.83	0.367
Female body condition	0.064 ± 0.056	1, 39.2	1.33	0.255
Female MDA	0.011 ± 0.010	1, 33.2	1.30	0.261
Female TAS	-0.225 ± 0.132	1, 57.4	2.90	0.094
Female tGSH	0.071 ± 0.072	1, 41.5	0.96	0.332
Haemolysis	0.396 ± 0.053	1, 114.0	55.73	<0.001*
Year	0.055 ± 0.163	1, 39.5	0.11	0.737
Final model				
Haemolysis	0.370 ± 0.044	1, 251.0	71.05	<0.001*
Nestling TAS				
Full model				
Hatching date	0.017 ± 0.020	1, 15.9	0.75	0.400
Brood size	0.001 ± 0.068	1, 18.1	0.00	0.993
Female body condition	-0.096 ± 0.087	1, 17.5	1.21	0.286
Female MDA	0.027 ± 0.015	1, 16.5	3.42	0.082
Female TAS	-0.117 ± 0.170	1, 37.4	0.47	0.495
Female tGSH	-0.016 ± 0.103	1, 18.5	0.03	0.874
Uric acid	0.113 ± 0.013	1, 73.6	71.92	<0.001*
Year	-0.011 ± 0.230	1, 16.7	0.00	0.961
Final model				
Hatching date	0.017 ± 0.006	1, 59.3	7.00	0.010*
Uric acid	0.116 ± 0.008	1, 169.0	226.37	<0.001*
Nestling tGSH				
Full model				
Hatching date	0.002 ± 0.034	1, 28.4	0.00	0.953
Brood size	-0.012 ± 0.115	1, 33.0	0.01	0.915
Female body condition	-0.179 ± 0.132	1, 28.7	1.83	0.187
Female MDA	-0.024 ± 0.023	1, 24.8	1.02	0.322
Female TAS	-0.258 ± 0.302	1, 50.3	0.73	0.397
Female tGSH	0.539 ± 0.170	1, 30.1	10.01	0.003*
Year	-0.029 ± 0.389	1, 29.6	0.01	0.941
Final model				
Female MDA	-0.035 ± 0.016	1, 55.5	4.80	0.033*
Female tGSH	0.279 ± 0.102	1, 83.5	7.42	0.008*

* Significant difference (α = 0.05)

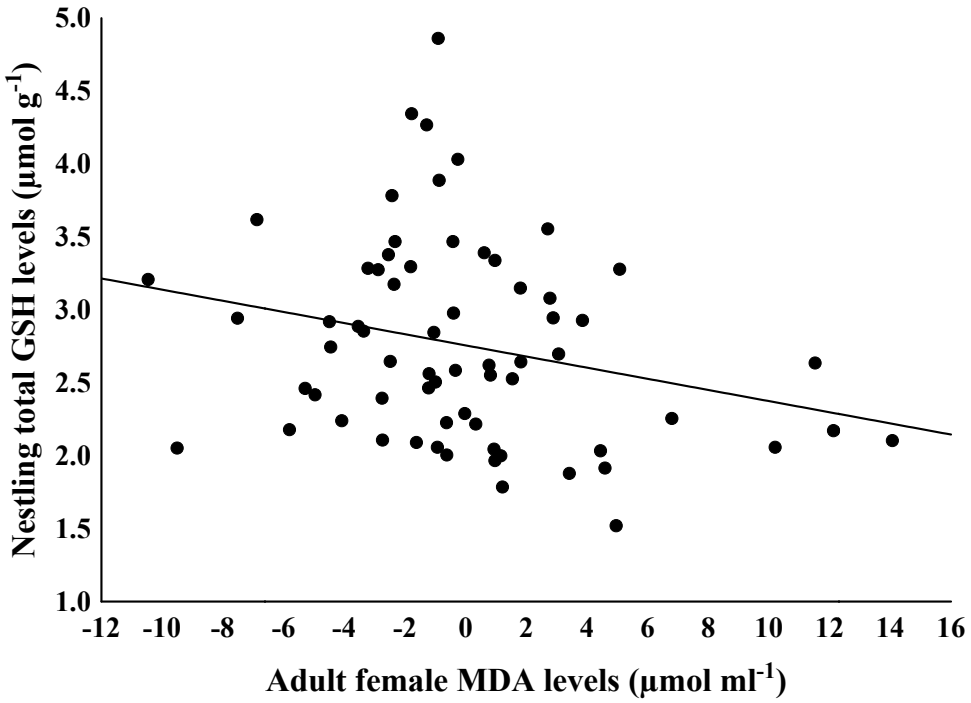

Figure 1. *Relation between nestling tGSH levels and adult female MDA levels, corrected by haemolysis degree ($F_{1, 64}$ = 4.36, p = 0.041) in the pied flycatcher.*

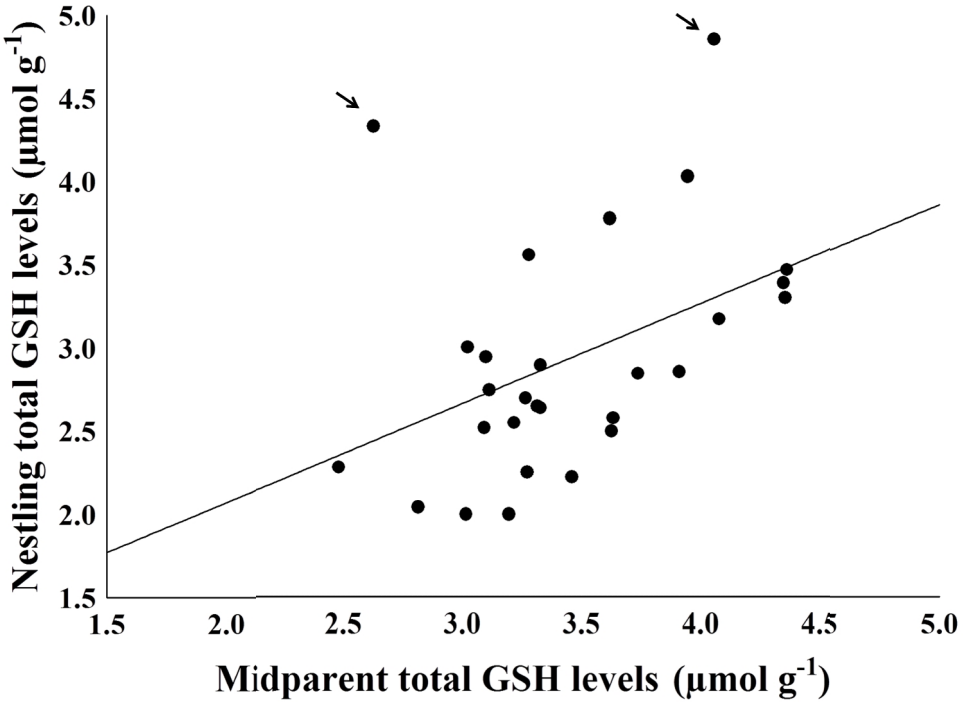

Figure 2. *Relation between midparent and mean nestling pied flycatcher tGSH levels in 2013 ($F_{1, 26}$ = 5.78, p = 0.024; y = 0.8758 + 0.5973x). Arrows indicate possible outliers (see "Results").*

Table 2. *Results of mixed models exploring associations among physiological variables and breeding variables (brood size and hatching date) of nestling pied flycatchers.*

	Estimate ± SE	*df*	*F*	*p*
MDA				
Full model				
Hatching date	0.014 ± 0.009	1, 111.0	2.33	0.129
Brood size	0.042 ± 0.034	1, 105.0	1.52	0.221
Sex	0.014 ± 0.045	1, 230.0	0.10	0.749
Body condition	0.018 ± 0.027	1, 208.0	0.44	0.507
Haemolysis	0.372 ± 0.044	1, 244.0	69.84	<0.001*
Year	0.105 ± 0.104	1, 129.0	1.03	0.311
Final model				
Haemolysis	0.371 ± 0.044	1, 250.0	70.53	<0.001*
TAS				
Full model				
Hatching date	0.006 ± 0.010	1, 75.6	0.35	0.558
Brood size	-0.020 ± 0.036	1, 76.9	0.32	0.571
Sex	0.014 ± 0.043	1, 136.0	0.11	0.735
Body condition	-0.014 ± 0.027	1, 179.0	0.25	0.616
Uric acid	0.116 ± 0.008	1, 156.0	214.15	<0.001*
Year	-0.149 ± 0.107	1, 104.0	1.95	0.166
Final model				
Uric acid	0.116 ± 0.008	1, 160.0	231.41	<0.001*
Year	-0.218 ± 0.071	1, 85.9	9.34	0.003*
tGSH				
Full model				
Hatching date	0.028 ± 0.020	1, 102.0	1.98	0.163
Brood size	-0.056 ± 0.071	1, 92.7	0.61	0.435
Sex	-0.186 ± 0.085	1, 238.0	4.81	0.029*
Body condition	0.025 ± 0.052	1, 234.0	0.24	0.628
Year	-0.215 ± 0.213	1, 126.0	1.02	0.315
Final model				
Hatching date	0.045 ± 0.012	1, 81.4	12.83	<0.001*
Sex	-0.185 ± 0.084	1, 240.0	4.80	0.029*

*Significant difference (α = 0.05)

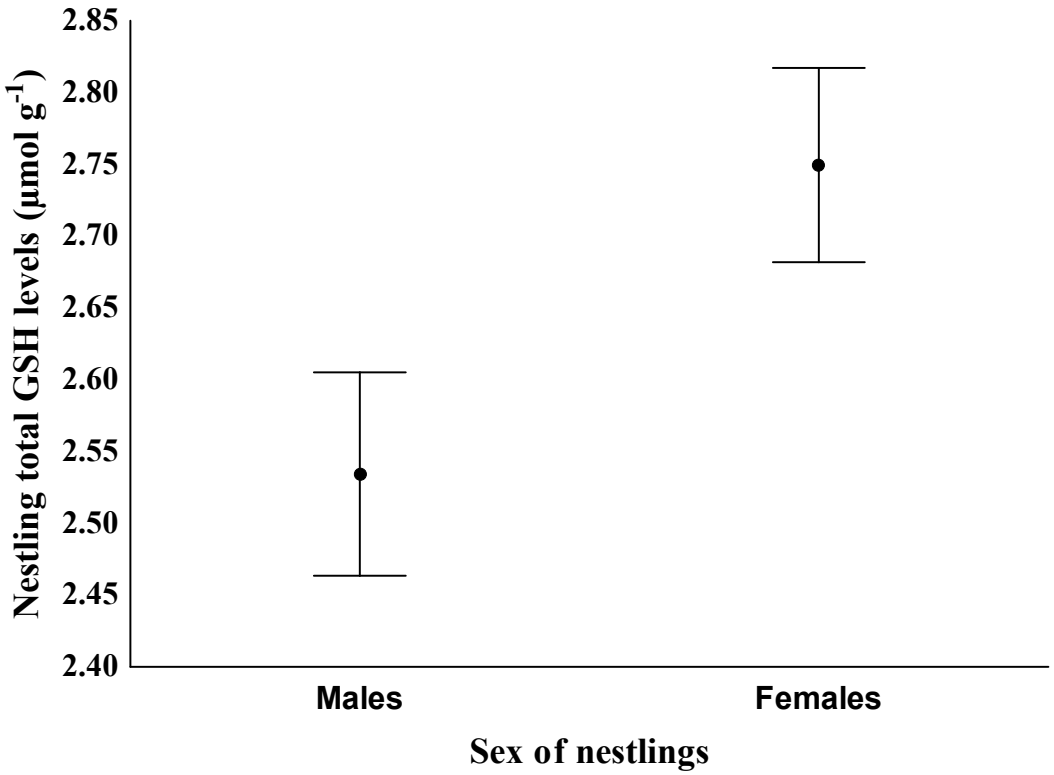

Figure 3. *Differences ($F_{1, 270}$ = 4.83, p = 0.029) in tGSH levels (mean ± SE) between female and male nestling pied flycatchers.*

DISCUSSION

We have searched for possible associations of nestling and parental oxidative variables and body condition in a pied flycatcher population. Moreover, we explored variation in levels of oxidative stress in nestlings in relation to sex and rearing conditions (hatching date and brood size). Our results showed that nestling tGSH levels were negatively associated with maternal MDA and positively associated with tGSH levels of both parents, also showing a high estimated heritability. Moreover, tGSH levels were higher in female than in male nestlings, and hatching date was positively associated with antioxidant defenses.

The negative association between nestling tGSH and MDA levels of the female parent could reflect costs and/or constraints derived from parental care. Females

experiencing lower levels of oxidative damage could be able to invest more resources in their offspring, for example, by increasing provisioning rates and/or supplying food items of higher nutritive value to nestlings. This could have a positive effect on nestling oxidative status, as reflected by higher constitutive levels of a key antioxidant, such as GSH. Alternatively, poorly fed nestlings would beg more intensely (Redondo & Castro, 1992; Cantarero *et al.*, 2013), which would increase their level of oxidative stress (increased oxidative damage and/or depleted antioxidant defenses; Moreno-Rueda *et al.*, 2012). This could elicit a behavioral response from parents, thereby increasing parental effort (Cantarero *et al.*, 2013), which could increase metabolic rate and oxidative stress (Nilsson, 2002; Alonso- Alvarez *et al.*, 2004; Wiersma *et al.*, 2004).

The strong positive correlation detected between parent-offspring tGSH levels could result from the relationship between parental oxidative status and parental effort and its subsequent effect on nestling antioxidant depletion. However, the high value of h^2 estimated for this trait suggests that the levels of this crucial antioxidant compound could be under strong genetic control. Similarly, a high heritability of GSH enzymes has been found for humans (60%; Chakraborty & Chaudhuri, 2001). The antioxidant role of GSH in RBCs depends on the rates of synthesis and degradation of GSH but also on the activity of enzymes involved in GSH action (Halliwell & Gutteridge, 2007; Lu, 2009). However, GSH is also involved in other important cellular processes not directly linked to antioxidant protection, such as regulation of DNA synthesis, melanogenesis, providing a reservoir for cysteine, or regulating cell growth and death (Lu, 2009). A strong genetic control of tGSH levels would help to buffer sudden changes in antioxidant requirements, assuring an optimal GSH supply to satisfy those alternative functions of this molecule.

Unlike tGSH, we found no heritability for TAS and MDA levels. TAS is a measure of the levels of nonenzymatic antioxidants -such as vitamins A, C, and E- and carotenoids (Cohen *et al.*, 2007), many of which are obtained from the diet. Thus, TAS could reflect parental ability to find high-quality food as well as other environmental factors affecting nutrient availability and allocation. Similarly, levels of MDA could be substantially influenced by environmental conditions, since oxidative damage reflects the balance between production of pro-oxidants and levels of antioxidant molecules, including those contributing to TAS (Costantini, 2014). Consistent with this, a recent study has also shown a high environmental contribution to MDA levels in nestlings of another passerine

(Losdat *et al.*, 2014). The lack of parent-offspring correlations in TAS and MDA levels could be also explained by the stronger effect of some rearing conditions (e.g., diet, nest ectoparasites; Rubolini *et al.*, 2006; De Coster *et al.*, 2012; López-Arrabé *et al.*, 2015; Wegmann *et al.*, 2015) in the nestlings as compared with adults.

It should be mentioned, however, that our conclusions on trait heritabilities must be taken with caution because they are based on midparent-offspring regressions, which could be influenced by shared environmental conditions that can affect similarities shown between relatives (Kruuk & Hadfield, 2007). The application of restricted maximum likelihood animal models to data from natural populations offers a powerful means of tackling these potentially confounding effects (Kruuk, 2004). Unfortunately, in our case, we had data from only two generations, which makes individual pedigrees unreliable.

Associations found between nestling antioxidant defenses (TAS and tGSH) and hatching date could be explained by seasonal changes in the availability of specific nutrients and/ or the metabolic constraints faced by the developing individual (Biard *et al.*, 2005; Sternalski *et al.*, 2010). Although early breeding is usually associated with higher parental and nestling quality (Verhulst & Nilsson, 2008 and references therein), it could also result in increased physiological stress (Lobato *et al.*, 2010) and metabolic costs as a result of cold weather conditions under which parental foraging efficiency is low (Stevenson & Bryant, 2000). Moreover, cooler microclimate conditions in the nest -as found in our study area early in the reproductive season- may impose higher metabolic costs for nestlings (Dawson *et al.*, 2005), which may have a direct negative impact on the oxidative status of nestlings that hatch earlier through depleting their antioxidant reserves (Bourgeon *et al.*, 2011).

Interestingly, we found significant differences in tGSH levels between nestling sexes, with these being higher in females than in males. Similar results have been shown for other species (Isaksson, 2013). This sexual difference is likely to be mediated via testosterone levels, because male nestlings often show higher circulating levels of this hormone than females (Naguib *et al.*, 2004; Müller *et al.*, 2007; Kozlowski & Ricklefs, 2011). Higher concentrations of testosterone could induce higher levels of oxidative stress (e.g., Alonso-Alvarez *et al.*, 2007, 2009; Mougeot *et al.*, 2009), leading to a faster decrease of tGSH in males as compared with females.

In conclusion, we have evaluated several natural correlates of oxidative stress levels in pied flycatcher nestlings, and we have found that sex and breeding time explain part of the variation in oxidative status, since antioxidant defenses were higher in female chicks and late-hatched broods. Moreover, we suggest that parental oxidative status and genes could be determinants for endogenous components of the nestlings antioxidant system, like tGSH levels. It has been demonstrated that early conditions can influence trade-offs and may program the individual phenotype throughout the life-time (Lindström, 1999; Metcalfe & Monaghan, 2001; Romero-Haro & Alonso-Alvarez, 2015). Understanding the relative contribution of genotype and environmental conditions to the oxidative stress experienced by the developing nestling -as well as the life-history trade-offs associated with the interaction between both factors- is essential to fully understand the importance of oxidative stress in shaping individual phenotype.

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ORNAMENTOS DEL PLUMAJE E INVERSIÓN REPRODUCTIVA EN RELACIÓN AL ESTADO OXIDATIVO

López-Arrabé, J., Cantarero, A., Pérez-Rodríguez, L., Palma, A. & Moreno, J. 2014. Plumage ornaments and reproductive investment in relation to oxidative status in the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae*). *Canadian Journal of Zoology*, 92:1019-1027

RESUMEN

Un aspecto clave en el estudio de los rasgos del plumaje con un papel potencial en la comunicación es el coste asociado con la producción y el mantenimiento de los ornamentos, expresado en términos de estrés oxidativo. En el Papamoscas cerrojillo (*Ficedula hypoleuca iberiae* Witherby, 1928), los machos y algunas hembras presentan un parche blanco en la frente y ambos sexos presentan bandas blancas conspicuas en las alas. Se examinaron las asociaciones entre estos ornamentos del plumaje y la capacidad individual para hacer frente al estrés oxidativo. Además, exploramos los costes oxidativos de la inversión reproductiva. La capacidad antioxidante total (TAS) en plasma y los niveles totales de glutatión (tGSH) en eritrocitos, así como un marcador de daño oxidativo en lípidos plasmáticos (malondialdehído, MDA) fueron medidos simultáneamente. Se encontraron asociaciones negativas entre los antioxidantes y los ornamentos en hembras incubadoras, aunque esta relación fue positiva durante la fase de cebas a polluelos. En machos, los niveles de MDA se asociaron negativamente con sus ornamentos, mientras que el TAS mostró una relación positiva. El MDA en hembras mostró una correlación positiva con el tiempo de incubación, mientras que esta relación fue negativa para los niveles de tGSH. Estos resultados indican que múltiples ornamentos del plumaje acromático parecen señalar la capacidad individual para hacer frente a los costes relacionados con el estrés oxidativo. Por otra parte, este estudio destaca el papel crítico de la incubación en el diseño de las estrategias vitales en aves.

ABSTRACT

A key aspect in the study of plumage traits with a potential role in communication is the cost associated with trait production and maintenance, expressed in terms of oxidative stress. In the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae* Witherby, 1928), males and some females exhibit a white forehead patch and both sexes present conspicuous white patches on the wings. We examined associations between these plumage ornaments and their ability to cope with oxidative stress. Furthermore, we explored oxidative costs of reproductive investment. Total antioxidant status (TAS) in plasma and glutathione (tGSH) levels in red blood cells, as well as a marker of oxidative damage in plasma lipids (malondialdehyde, MDA), were assessed simultaneously for the first time in studies of avian reproduction. We found negative associations between antioxidants and ornaments in incubating females, although this relationship was positive while feeding nestlings. For males, MDA levels were negatively associated with ornaments, while TAS showed a positive relation. Female MDA showed a positive correlation with intensity of incubation attendance, while this relation was negative for tGSH levels. These results indicate that multiple achromatic plumage ornaments signal the individual capacity to cope with costs related to oxidative stress. Moreover, this study highlights the critical role of incubation for avian life histories.

Keywords: egg attendance, *Ficedula hypoleuca iberiae*, glutathione, malondialdehyde (MDA), Iberian Pied Flycatcher, provisioning rates, total antioxidant status (TAS), achromatic plumage patches.

INTRODUCTION

Ornamental plumage in birds is one of the most studied types of sexually selected traits (Hill & McGraw, 2006) and often shows condition-dependent variation (Cotton *et al.*, 2004 and references therein). Plumage colour may be due to pigmentation, feather structure, or a combination of both (McGraw *et al.*, 2002). Although feather colouration can be produced by various pigments, melanins are the most prevalent and can also be deposited in all avian integuments (Prum & Williamson, 2002; Leskinen *et al.*, 2012). However, apart from these colouration traits, some plumage ornaments consist of patches of white plumage that produce a scattering of light in all directions by unmelanized feather keratin (Prum *et al.*, 1999). Perhaps because the production of achromatic plumage requires neither pigments nor a precisely ordered feather nanostructure, producing a small amount of unmelanized feathers has traditionally been considered to be noncostly in terms of resource allocation. However, proposed mechanisms for maintaining the honesty of unpigmented signals have usually focused not on production costs but on various costs of maintaining the trait (McGlothlin *et al.*, 2007), such as a higher risk of physical abrasion (e.g., Barrowclough & Sibley, 1980) or biotic degradation by feather-degrading bacteria or ectoparasites (Kose *et al.*, 1999; Goldstein *et al.*, 2004; Gunderson *et al.*, 2008; Burtt *et al.*, 2011; Ruiz-De-Castañeda *et al.*, 2012).

A key aspect in the study of the function and evolution of plumage traits with a potential role in communication is the cost associated with trait production and maintenance. Handicap Theory predicts that the honesty of sexual ornaments reflects individual capacity to withstand signalling costs (Zahavi, 1977; Andersson & Iwasa, 1996). These costs have been traditionally associated with resource limitation (Siefferman & Hill, 2007), impaired immunocompetence (Folstad & Karter, 1992), or energetic constraints (Hill, 2000). However, more recently, some of these costs have been considered in terms of oxidative stress (Von Schantz *et al.*, 1999; Alonso-Alvarez *et al.*, 2007). Oxidative stress is usually defined as the imbalance between levels of reactive oxygen and nitrogen species and the state of the antioxidant machinery in the organism (Halliwell & Gutteridge, 2007; Metcalfe & Alonso-Alvarez, 2010). Oxidative stress may

lead to oxidative damage in important biomolecules (lipids, proteins, and DNA), which could impair their functionality (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2007). However, research on oxidative costs of sexual signalling has been foremost focused on carotenoid-based ornaments (Pérez-Rodríguez, 2009; Simons *et al.*, 2012) and melanin-based ornaments (e.g., Galván & Alonso-Alvarez, 2008; Roulin *et al.*, 2011), but little is known about achromatic ornaments. Apart from the particular case of signal expression, oxidative stress has important health-related implications (Costantini *et al.*, 2006) and is considered to be a mediator of life-history trade-offs between growth, reproduction, and self-maintenance (Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe & Alonso-Alvarez, 2010). For instance, there is evidence that nestling provisioning rates, the most frequently used measure of parental effort (Moreno *et al.*, 1999), are related to high metabolic rates (Moreno *et al.*, 2001; Nilsson, 2002) and these high levels of metabolism may cause oxidative stress by the increased production of pro-oxidant metabolites and free radicals (Von Schantz *et al.*, 1999).

In the Iberian Pied Flycatcher (*Ficedula hypoleuca iberiae* Witherby, 1928), a small passerine bird, males and females differ in the expression of melanin-based dorsal plumage; males are conspicuously black, while females are brown (Lundberg & Alatalo, 1992). In Iberian populations, males and some females exhibit a distinctive white forehead patch (Potti, 1993; Morales *et al.*, 2007) and both sexes have conspicuous white patches on their wings (Lundberg & Alatalo, 1992). A large number of studies have shown that the presence and extent of these white-plumage patches in both sexes of Pied Flycatchers are associated with reproductive success (Morales *et al.*, 2007), resistance to infections (Potti & Merino, 1996), physiological stress (Lobato *et al.*, 2010), hormonal levels (Moreno *et al.*, 2014), or genetic quality (related Collared Flycatchers (*Ficedula albicollis* Temminck, 1815); Török *et al.*, 2003). Likewise, blackness of males has shown associations with timing of breeding and reproductive success (Galván & Moreno, 2009; Sirkiä *et al.*, 2010), predation risk (Slagsvold *et al.*, 1995), condition (Slagsvold & Lifjeld, 1992), and immune response (Kerimov *et al.*, 2013). White-plumage patches (but not plumage blackness) of flycatchers have been also related to individual and mate oxidative stress in the genus *Ficedula* Brisson, 1760 (Marko *et al.*, 2011; Moreno *et al.*, 2011, 2013*a*, 2013*b*).

Our main goal was to examine possible associations between plumage sexual ornaments of Iberian Pied Flycatchers and their oxidative status during the reproductive period. Previous studies have shown that the sign of relationships between reproduction and oxidative status can change depending on reproductive phase because oxidative stress can reflect both costs of and constraints on reproduction (Stier *et al.*, 2012). For these reasons, we aim to study the covariation between oxidative traits and before and after hatching reproductive investment in this species, something which has not been previously done. To properly evaluate the redox balance of individuals, measures of antioxidant capacity and oxidative damage must be obtained simultaneously (Costantini & Verhulst, 2009; Monaghan *et al.*, 2009; Pérez-Rodríguez, 2009). Here we have used plasma malondialdehyde (MDA) levels, a by-product of peroxidative decomposition of unsaturated lipids (Halliwell & Gutteridge, 2007), as a measure of oxidative damage and a presumptive marker of oxidative stress (Mateos *et al.*, 2005; Halliwell & Gutteridge, 2007; Sepp *et al.*, 2012). To monitor antioxidant defences, we have measured simultaneously the total antioxidant status (TAS) of plasma and total glutathione (tGSH) levels in red blood cells. TAS measures the pooled effect of all extracellular antioxidant compounds of the blood (Miller *et al.*, 1993; Cohen *et al.*, 2007; Costantini, 2011). Glutathione is a tripeptide thiol found in animal cells functioning in the protection of cells against free radicals, being often considered to be the most important intracellular antioxidant (Meister, 1991; Wu *et al.*, 2004). No previous study has, to our knowledge, included these three variables simultaneously in analyses of avian ornamentation and reproductive investment.

Given the potential costs of investment in ornamentation and reproduction in terms of oxidative stress, we predicted that (*i*) higher levels of signalling (i.e., larger achromatic patches in both sexes or more melanised dorsal plumage in males) would be related to a higher capacity to cope with oxidative stress (more antioxidant defences and (or) lower levels of oxidative damage), and (*ii*) there would be a relationship between reproductive investment (incubation attendance, provisioning rates) and oxidative status of breeding individuals. However, in a correlative study such as this, causes and effects are difficult to separate, preventing us from making unique predictions. Thus, if higher oxidative stress limits investment in reproduction, then we would predict negative relationships between reproductive investment and oxidative stress, whereas the

opposite is expected if higher oxidative levels result from increased reproductive investment. Moreover, the sign and intensity of these associations could change between the incubation and the nestling phases as mentioned above.

METHODS

General field methods

The study was conducted during the springs of 2012 and 2013 in a montane forest of Pyrenean oak (*Quercus pyrenaica*) at 1200 m above sea level in Valsaín, central Spain (40°54´N, 04°01´W), where long-term studies on cavity-nesting birds have been ongoing since 1991. In the study area, there are 570 nest boxes (for dimensions, structure, and placement of nest boxes see appendix in Lambrechts *et al.*, 2010) occupied by Iberian Pied Flycatchers, Great Tits (*Parus major*), Eurasian Nuthatches (*Sitta europaea*) and Blue Tits (*Cyanistes caeruleus*).

We followed breeding activities from the early stages of nest construction to fledging in nest boxes occupied by Iberian Pied Flycatchers. Egg laying in the Iberian Pied Flycatcher population under study typically begins in late May and modal clutch size is six and chicks usually fledge at the age of 17 days. The female incubates and broods alone and receives part of her food from her mate (Moreno *et al.*, 2011). Breeding activities were followed routinely and laying and hatching dates and brood sizes at fledging were determined.

In 2012 and 2013, males and females were captured in their nest boxes with traps while provisioning nestlings of 7-8 days, ringed if necessary, or identified and weighed to the nearest 0.01 g with a digital balance. As experiments were carried out in 2012 and 2013, we have only included unmanipulated control birds in this study. We took a blood sample of about 120 µl from the brachial vein that was collected in heparinized microcapillaries. Females were blood-sampled in 2012 (N = 37) and 2013 (N = 33), whereas males were only blood-sampled in 2013 (N = 32). Blood samples were stored in Eppendorf tubes in an icebox until returning to the laboratory in the same day.

Plasma was separated from blood by centrifugation (10 min at 12000 rpm) and then both fractions were stored at -80 °C until analysed for assaying MDA, TAS, and uric acid from plasma, and tGSH in red blood cells (see below). If haemolysis occurs during sampling, then a possible efflux of intracellular pro-oxidants and antioxidant molecules into plasma could alter levels of oxidative markers measured in blood samples, thereby confounding interpretation of results. Thus, haemolysis levels in plasma samples were noted by a visual detection of red colour of plasma, as a consequence of release of haemoglobin from red blood cells, in a continuous gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree to minimize interobserver variability.

Additionally, in 2013 some females belonging to this sample (N = 30) were also captured during incubation (7 or 8 days after clutch completion) in the nest box during daytime without traps, as they are not easily frightened away from the nest when incubating. They were ringed if necessary, identified, weighed, and blood-sampled in the same way as above. The whole procedure took less than 5 min and no female deserted after manipulation. All captured individuals were photographed at the nestling stage to analyze their ornamentation: head from above with a ruler below the chin for reference and folded wing from above with ruler beside for reference (Moreno *et al.*, 2011). Then, we determined areas of forehead and wing white patches analyzing photographs with Photoshop CS4 (version 11.0; Adobe Inc., San Jose, California, USA) according to Moreno *et al.* (2014). In the case of males, we also determined the blackness of dorsal plumage as the percentage of black feathers on the back of the head and mantle (Galván & Moreno, 2009).

Lipid peroxidation assays

Plasma concentrations of MDA were calculated following Agarwal & Chase (2002) with some modifications made by Mougeot *et al.* (2009). Assays were carried out in 2 ml capacity screw-top microcentrifuge tubes and all chemical solutions were prepared using ultra-pure water (Milli-Q Synthesis; Millipore, Watford, UK). For calibration, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane stock solution (10 M in 40% ethanol) serially diluted using 40% ethanol. Twenty-five microlitres butylated

hydroxytoluene solution (0.05% m/v in 95% ethanol), 200 µl phosphoric acid solution (0.44 M), and 50 µl thiobarbituric acid solution (42 M) were added to 25 µl of plasma samples (1 : 2.5 dilution in water) or standards. Samples were mixed using a vortex for 5 s and then heated at 100 °C for 1 h on a dry bath incubator to allow formation of MDA-TBA adducts. The reaction was then stopped by placing samples on ice for 5 min before 125 µl *n*-butanol was added and tubes were mixed using a vortex for 1 min. Tubes were then centrifuged at 14000 rpm and 4 °C for 3 min, before the upper (*n*-butanol) phase was collected and transferred into a high-performance liquid chromatography (HPLC) vial for analysis. Samples (10 µl) were injected into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, California, USA) fitted with a 5 µm ACE guard column and 5 µm ODS 100 mm × 4.6 mm column (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland) maintained at 37 °C. The mobile phase was methanol-buffered (40 : 60 v/v), the buffer being a 50 mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 ml min^{-1}. Data were collected using a fluorescence detector (reference No. G1321A; Agilent Technologies) set at 515 nm (excitation) and 553 nm (emission). Repeatability showed by a set of samples assayed in duplicate and TEP standards was high (R = 0.722, N = 66, p < 0.001 and R = 0.962, N = 6, p < 0.001, respectively).

Total antioxidant status (TAS)

TAS was assayed following Miller *et al.* (1993) with some modifications made by Cohen *et al.* (2007). Metmyoglobin was generated by mixing equal volumes of 400 µM myoglobin (reference No. M0630-250MG; Sigma-Aldrich, St. Louis, Missouri, USA) and 740 µM potassium ferricyanate, then passing the mixture through a column of Sephadex (reference No. G15-120; Sigma-Aldrich, St. Louis, Missouri, USA). The chromogen, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid, ABTS), was mixed in phosphate-buffered saline (PBS) to 153 µM. The standard was made by dissolving a water-soluble α-tocopherol derivative, Trolox, in PBS to 1.7 mM. The assay was run in 96-well flat-bottomed clear microplates on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont, USA). Temperature was maintained at 37 °C and

readings were taken at 660 nm. Only one 12-well row was used from the plate at a time. Five microlitres of standard (Trolox) or samples were put separately into the wells. Next, 15 µl metmyoglobin and 250 µl ABTS were sequentially added to each well. A multichannel pipette was used to simultaneously add 50 µl of 300 µM H_2O_2 to all the wells, starting the reaction. Kinetic measurements using the spectrophotometer were taken at 10 s intervals; readings were synchronized to the start of the reaction (i.e., injection of H_2O_2) manually using a timer. The reaction runs for around 10 min. Most of samples were assayed in duplicate and showed high repeatability (R = 0.981, N = 60, p < 0.001).

Intracellular total glutathione (tGSH) level

tGSH levels in red blood cells were determined as described in López-Arrabé *et al.* (2014). Briefly, samples of red blood cells were homogenized in a stock buffer and mixed with 10% trichloroacetic acid. The mixture was mixed using a vortex, centrifuged, and the supernatant was separated. Three working solutions were made up in a reaction buffer as follows: (1) 0.3 mM NADPH, (2) 6 mM DTNB, and (3) 50 U ml^{-1} GSH reductase. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.). To 75 µl of sample (supernatant), we added 240 µl of the mixture of solutions 1 and 2. Afterward, 20 ml of solution 3 was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH. A set of samples assayed in duplicate showed high repeatability (R = 0.983, N = 106, p < 0.001).

Measurement of levels of uric acid

Uric acid is the main form of nitrogen excretion in birds and an indicator of amino acid catabolism. But, in addition, uric acid is also a powerful antioxidant whose concentration is frequently positively related to TAS values (Cohen *et al.*, 2007; Hõrak *et al.*, 2007; Pérez-Rodríguez *et al.*, 2008), potentially confounding the interpretation of this marker (Cohen *et al.*, 2007; Costantini, 2011). For this reason TAS values corrected for uric acid

are recommended over raw TAS levels (Cohen *et al.*, 2007). The uricase/peroxidase method was used for measuring levels (kit reference No. 11522; Biosystems, Barcelona, Spain) and using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.). Reagent volumes and further assay details were implemented according to manufacturer instructions. A set of samples assayed in duplicate showed high repeatability (R = 0.99, N = 45, $p < 0.001$).

Behavioural data

In 2012 and 2013, 5 days after clutch completion (day 6 of incubation) and 2 and 8 days after hatching date, we recorded nest activity inside nest boxes for about 95 min (mean ± SE: 97.28 ± 1.83 min, N = 110) with a cold white light (LED; 5 mm) powered by a 3 V battery and a camera (GoPro HD Hero1) mounted on the roof inside the nest box (Cantarero *et al.*, 2013). From records taken during incubation, we estimated the proportion of time spent by the female inside the nest box or "egg attendance", which includes the time allocated to incubating and turning the eggs (Cantarero *et al.*, 2013). In addition, we estimated hourly provisioning rates by males to incubating females. From films taken during the early and late nestling periods, we obtained hourly provisioning rates by males and females to nestlings. At the early nestling phase, we also estimated "brooding attendance", which is the proportion of time spent by the female inside the nest box.

Statistical analyses

Physiological variables were normally distributed and were therefore analyzed with general linear models (GLM) using the STATISTICA version 8.0 package (StatSoft, Inc., Tulsa, Oklahoma, USA) and final models were selected by backward elimination of nonsignificant terms to improve the models.

To establish differences in oxidative parameters, we used Student´s *t*-test analyses between males and females and between reproductive phases. Moreover, we explored correlation matrices for possible associations between these variables.

In both males and females, the association between wing and forehead patches was weak (males: r^2 = 0.03; females: r^2 = 0.13). To determine if incubating female oxidative parameters were related to their own ornaments, we applied GLM models with MDA, TAS, or tGSH levels measured in incubation as dependent variables and laying date, clutch size, incubating female body mass, female forehead and wing white patches as covariates. For female physiological parameters measured during the nestling phase, we applied similar models but substituted laying date, clutch size, and incubating female body mass with hatching date, brood size at the age of 9 days, and female body mass during the nestling phase, respectively, and included year as fixed factor. Since Moreno *et al.* (2013*a*) found a strong correlation between oxidative damage levels of females and blackness of their mates, we included this male trait in female MDA models. In the case of males, models included physiological variables as dependents variables, while hatching date, brood size, male body mass, blackness of dorsal plumage, and male forehead and wing white patches were introduced as covariates.

We analyzed if female oxidative parameters during incubation were related to incubating behaviour, including laying date, clutch size, egg attendance, and male provisioning rate during incubation as covariates. During the nestling phase, we analyzed the associations between parental care and physiological condition for both females and males. For females, we included oxidative variables as dependent variables, year as fixed factor, and brooding attendance and provisioning rates by females to nestlings as covariates. In the case of males, models included oxidative parameters as dependents and male provisioning rates to nestlings as covariates. In both cases, we controlled by hatching date and brood size.

In all cases, we controlled MDA, TAS, and tGSH models by including haemolysis degree and TAS analyses by levels of uric acid (see above) as covariates.

RESULTS

There were no differences between male and female oxidative levels (Student´s *t*-test: all $p > 0.05$; Table 1) and between reproductive phases for female variables (Student´s *t*-

test: all $p > 0.05$; Table 1). No correlations were found between both female and male MDA, TAS, and tGSH levels (all $p > 0.05$). In all cases, haemolysis degree was positively correlated with MDA levels (Tables 2, 3, 4) and only for females during the nestling stage was haemolysis negatively correlated with tGSH levels (Tables 2, 3, 4). In all cases, levels of uric acid were positively correlated with TAS values (Tables 2, 3, 4).

Oxidative status and plumage ornamentation

At incubation female TAS and tGSH levels were negatively related to folded wing-patch area and forehead white patch, respectively (Table 2). tGSH was higher in heavier females (Table 2).

While feeding nestlings, females showed a positive association between TAS and presence of a forehead patch (Table 3). Females that bred earlier showed higher levels of TAS (Table 3). While tGSH level was once again positively related to female body mass, it showed a negative association with the number of nestlings (Table 3). Female MDA levels were not related to ornamental traits or condition at any stage (Tables 2, 3).

Table 1. *Levels (mean ± SE) of malondialdehyde (MDA), total antioxidant status (TAS) of plasma, and total glutathione (tGSH) in male and female Iberian Pied Flycatchers during the incubation and nestling phases.*

Sex	Variable	Incubation	Nestling phase
Females	MDA	10.00 ± 0.77	11.21 ± 0.64
	TAS	2.60 ± 0.16	1.62 ± 0.08
	tGSH	3.36 ± 0.16	3.27 ± 0.09
Males	MDA	-	14.24 ± 1.15
	TAS	-	1.83 ± 0.08
	tGSH	-	3.55 ± 0.12

In the case of males, wing-patch area was negatively and positively associated with MDA and tGSH levels, respectively (Table 4), while TAS showed no associations with

ornaments (Table 4). MDA levels also showed a positive correlation with brood size (Table 4).

Table 2. *Results of GLM for female physiological variables (MDA, TAS and tGSH) measured during the incubation phase of the Iberian Pied Flycatcher.*

	df	*β*	*F*	*p*	*Partial η²*
MDA					
Full model					
Laying date	1, 13	-0.093	0.194	0.667	0.015
Clutch size	1, 13	0.297	1.762	0.207	0.119
Body mass	1, 13	0.317	2.806	0.118	0.177
Forehead patch area	1, 13	0.057	0.072	0.792	0.005
Wing patch area	1, 13	0.062	0.081	0.780	0.006
Male Blackness	1, 13	-0.070	0.098	0.759	0.007
Haemolysis	1, 13	0.484	5.126	0.041*	0.283
Minimal model					
Haemolysis	1, 25	0.609	14.761	0.001*	0.371
TAS					
Full model					
Laying date	1, 10	0.088	0.220	0.649	0.022
Clutch size	1, 10	-0.004	<0.001	0.984	<0.001
Body mass	1, 10	0.153	0.737	0.411	0.069
Forehead patch area	1, 10	0.133	0.485	0.502	0.046
Wing patch area	1, 10	-0.495	5.534	0.040*	0.356
Uric acid	1, 10	0.910	22.748	<0.001*	0.695
Haemolysis	1, 10	-0.088	0.234	0.639	0.023
Minimal model					
Wing patch area	1, 17	-0.397	8.517	0.010*	0.334
Uric acid	1, 17	0.915	45.375	<0.001*	0.727
tGSH					
Full model					
Laying date	1, 18	0.302	2.692	0.118	0.130
Clutch size	1, 18	0.169	0.745	0.399	0.039
Body mass	1, 18	0.411	5.495	0.031*	0.234
Forehead patch area	1, 18	-0.403	4.513	0.048*	0.200
Wing patch area	1, 18	-0.116	0.370	0.550	0.020
Haemolysis	1, 18	-0.362	3.199	0.090	0.151
Minimal model					
Body mass	1, 24	0.339	4.333	0.048*	0.153
Forehead patch area	1, 24	-0.489	9.014	0.006*	0.273

* Significant difference ($\alpha = 0.05$)

Table 3. Results of GLM for female physiological variables (MDA, TAS and tGSH) measured during the nestling phase of the Iberian Pied Flycatcher.

	df	*β*	*F*	*p*	*Partial η²*
MDA					
Full model					
Hatching date	1, 37	-0.155	0.388	0.537	0.010
Brood size	1, 37	0.004	<0.001	0.977	<0.001
Body mass	1, 37	-0.010	0.004	0.949	<0.001
Forehead patch area	1, 37	-0.006	0.001	0.970	<0.001
Wing patch area	1, 37	-0.216	1.681	0.203	0.043
Male blackness	1, 37	0.028	0.031	0.860	<0.001
Haemolysis	1, 37	0.440	6.687	0.014*	0.153
Year	1, 37	-0.005	<0.001	0.984	<0.001
Minimal model					
Haemolysis	1, 65	0.491	20.595	<0.001*	0.241
TAS					
Full model					
Hatching date	1, 25	-0.499	4.865	0.037*	0.162
Brood size	1, 25	-0.176	1.975	0.172	0.073
Body mass	1, 25	0.185	1.630	0.213	0.061
Forehead patch area	1, 25	0.312	6.484	0.017*	0.206
Wing patch area	1, 25	0.087	0.392	0.537	0.015
Uric acid	1, 25	0.756	35.067	<0.001*	0.584
Haemolysis	1, 25	0.034	0.079	0.780	0.003
Year	1, 25	-0.356	2.128	0.157	0.078
Minimal model					
Hatching date	1, 34	-0.213	4.569	0.039*	0.119
Forehead patch area	1, 34	0.211	4.143	0.049*	0.109
Uric acid	1, 34	0.824	63.494	<0.001*	0.651
tGSH					
Full model					
Hatching date	1, 51	0.004	<0.001	0.984	<0.001
Brood size	1, 51	-0.289	5.340	0.024*	0.095
Body mass	1, 51	0.426	9.713	0.003*	0.160
Forehead patch area	1, 51	0.013	0.011	0.917	<0.001
Wing patch area	1, 51	0.154	1.224	0.274	0.023
Haemolysis	1, 51	-0.354	7.162	0.010*	0.123
Year	1, 51	-0.165	0.684	0.412	0.013
Minimal model					
Brood size	1, 61	-0.309	7.228	0.009*	0.106
Body mass	1, 61	0.308	6.901	0.011*	0.102
Haemolysis	1, 61	-0.272	5.516	0.022*	0.083

* Significant difference (α = 0.05)

Oxidative status and reproductive investment

During incubation, female MDA showed a positive correlation with intensity of egg attendance ($F_{1,21}$ = 6.088, p = 0.022, partial η^2 = 0.225; Figure 1), although it was not related to male provisioning rate to females ($F_{1,17}$ = 1.358, p = 0.260, partial η^2 = 0.074). While tGSH levels negatively covaried with egg attendance ($F_{1,24}$ = 7.445, p = 0.012, partial η^2 = 0.237; Figure 2), this was not related to male provisioning rate to females ($F_{1,18}$ = 0.659, p = 0.427, partial η^2 = 0.035). TAS levels of incubating females were not related to attendance variables (all p > 0.05).

Neither female nor male oxidative parameters were related to parental-care investment during the nestling phase (all p > 0.05).

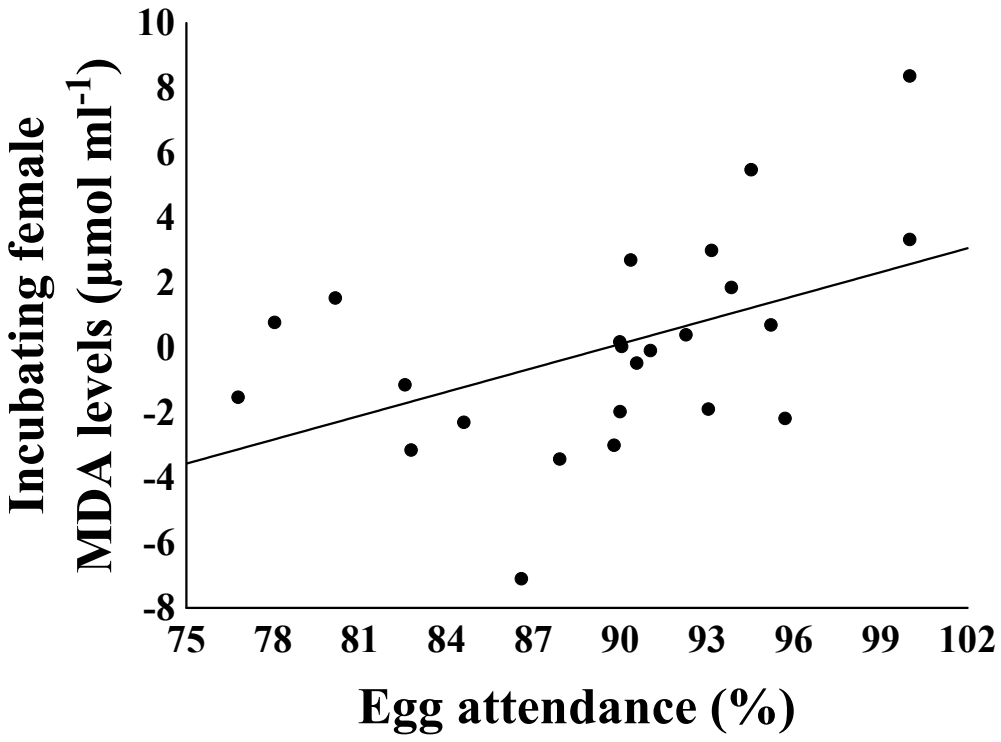

Figure 1. *Association between residuals of female Iberian Pied Flycatcher MDA levels measured during incubation (corrected by haemolysis degree) and egg attendance.*

Table 4. Results of GLM for male physiological variables (MDA, TAS and tGSH) measured during the nestling phase of the Iberian Pied Flycatcher.

	df	*β*	*F*	*p*	*Partial η²*
MDA					
Full model					
Hatching date	1, 21	0.010	0.008	0.930	<0.001
Brood size	1, 21	0.291	5.212	0.033*	0.199
Body mass	1, 21	0.086	0.495	0.489	0.023
Blackness	1, 21	-0.079	0.379	0.545	0.018
Forehead patch area	1, 21	0.024	0.036	0.852	0.002
Wing patch area	1, 21	-0.245	4.065	0.057	0.162
Haemolysis	1, 21	0.626	19.119	<0.001	0.476
Minimal model					
Brood size	1, 25	0.296	7.614	0.011*	0.233
Wing patch area	1, 25	-0.261	5.533	0.027*	0.181
Haemolysis	1, 25	0.627	30.163	<0.001*	0.547
TAS					
Full model					
Hatching date	1, 16	-0.172	1.788	0.200	0.100
Brood size	1, 16	0.089	0.387	0.542	0.024
Body mass	1, 16	0.073	0.327	0.575	0.020
Blackness	1, 16	-0.176	1.680	0.213	0.095
Forehead patch area	1, 16	0.233	3.207	0.092	0.167
Wing patch area	1, 16	0.194	2.535	0.131	0.137
Uric acid	1, 16	0.606	14.520	0.001*	0.476
Haemolysis	1, 16	0.202	1.718	0.208	0.097
Minimal model					
Uric acid	1, 23	0.833	52.357	<0.001*	0.695
tGSH					
Full model					
Hatching date	1, 23	-0.032	0.037	0.849	0.001
Brood size	1, 23	0.237	1.656	0.211	0.067
Body mass	1, 23	-0.171	0.929	0.345	0.039
Blackness	1, 23	-0.240	1.688	0.207	0.068
Forehead patch area	1, 23	0.242	1.706	0.204	0.069
Wing patch area	1, 23	0.478	7.382	0.012*	0.243
Haemolysis	1, 23	-0.130	0.364	0.552	0.015
Minimal model					
Wing patch area	1, 29	0.554	12.863	0.001*	0.307

* Significant difference ($\alpha = 0.05$)

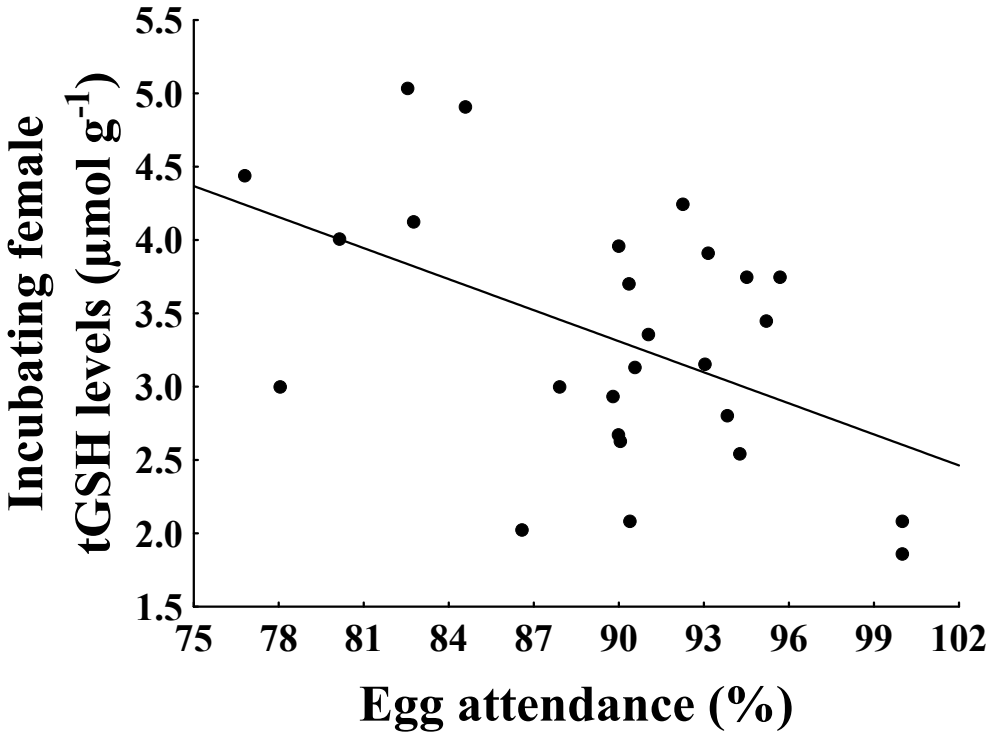

Figure 2. Association between female Iberian Pied Flycatcher tGSH measured during incubation and egg attendance.

DISCUSSION

We have conducted an observational study to establish possible associations between oxidative measures, plumage ornamentation, and parental-care behaviours in breeding individuals of an Iberian Pied Flycatcher population. Our results show that female antioxidant defences were associated with the expression of multiple achromatic plumage ornaments. Both measured ornaments, forehead and wing white patches, were correlated with tGSH and TAS. However, the sign of the association between ornamentation and tGSH in females differed between reproductive stages, being negative during the incubation phase and positive at the nestling stage. Males with larger wing patches also showed lower levels of oxidative stress. On the other hand, oxidative status of incubating female was related to egg-attendance behaviour, although there was a lack of association between oxidative stress parameters of both males and females and their provisioning rates at any stage of nestling care.

Evidence for an adaptive function of sexual signalling in females has been based on positive relationships between ornament expression and traits indicative of quality (Kraaijeveld *et al.*, 2007; Morales *et al.*, 2008). Patches of white plumage in females could express a metabolically and oxidatively costly female strategy to obtain resources necessary for breeding (Rosvall, 2011; Moreno *et al.*, 2013*b*), which may be beneficial in highly competitive circumstances (Midamegbe *et al.*, 2011). Thus, less ornamented females could suffer less physiological costs while breeding, but could also suffer competitive exclusion from breeding resources in some circumstances. Recently, it has been shown in Iberian Pied Flycatchers that female-female competition is influenced by forehead-patch expression (Morales *et al.*, 2014) and that the area of the wing white patch in females is associated with higher circulating testosterone levels during the incubation phase (Moreno *et al.*, 2014) that can lead to weaker resistance to oxidative damage (Alonso-Alvarez *et al.*, 2007; Mougeot *et al.*, 2009). Moreover, Moreno *et al.* (2013*b*) showed that the addition of a forehead white patch to Iberian Pied Flycatcher females without one leads to increased oxidative damage. Although we did not find associations between female ornaments and oxidative damage during incubation, our results are in accordance with these assumptions by indicating that females with larger ornaments experience a depletion in antioxidant defences at the beginning of the reproductive period possibly due to female-female competitive interactions. This depletion of antioxidants could result in higher levels of oxidative stress. During the nestling phase, competitive interactions between females may be rare and ornamented females would express their capacity to cope with increased levels of oxidative stress. Alternatively, the contrasting patterns of association between female achromatic ornaments and antioxidant capacity at both phases of the reproductive period could be mediated by the capacity of the female to allocate resources to egg production. Thus, highly ornamented females would be able to invest relatively higher amounts of antioxidants into eggs, thus down-regulating their own circulating defences, but quickly recover afterwards, during the nestling period. Consistent with this possibility, Potti *et al.* (2013) demonstrated that forehead patches of female Iberian Pied Flycatchers are related to some fitness advantages, as increased fecundity, thereby compensating the costs of ornament expression, supporting the possibility that more ornamented females are indeed of higher quality than those with smaller ornaments. Finally, in contrast to Moreno *et al.* (2013*a*), we have detected no relationship between oxidative damage of

females and plumage darkness of males, possibly due to a lack of males with percentages of black plumage below 50% in the study years compared with the year of the former study.

The size of the white forehead patch of males, but not wing patch size, has been shown to be positively associated with plasma antioxidant capacity, although not with oxidative damage during the nestling phase, in the closely related Collared Flycatcher (Marko *et al.*, 2011). Moreover, Moreno *et al.* (2011) showed a similar relationship between forehead-patch size and antioxidants, as well as a negative covariation between levels of MDA and this ornament in males of Iberian Pied Flycatchers. In our study, we found such associations between oxidative damage, glutathione levels, and wing ornamentation, suggesting that multiple plumage ornaments act as a signalling complex that provide information about individual oxidative status, decreasing the likelihood that individual quality will be improperly assessed (Guindre-Parker *et al.*, 2013). Which plumage trait (forehead or wing patch) turns up significant may depend on the amount of variation in the different traits present in the sample studied. Male achromatic signals may function both as mating signals (Hegyi *et al.*, 2010) and as social signals in territorial contexts (Järvistö *et al.*, 2013). Our study confirms previous results, with multiple measures of oxidative status, that achromatic signals may reveal the capacity to cope with oxidative stress in these birds.

Females that bred earlier showed higher levels of antioxidants. Although early breeding is usually associated with individual quality (Verhulst & Nilsson, 2008 and references therein), producing eggs early in the season could also result in an increase in physiological stress (Lobato *et al.*, 2010) and in energetic costs due to cold weather conditions under which foraging efficiency is low (Stevenson & Bryant, 2000), and this might have negative effects on current and future reproductive success (Te Marvelde *et al.*, 2012). This factor combined with costs derived from signalling involves stressful conditions for females that might thereby compromise their self-maintenance. Thus, our results are consistent with the idea that only those females that are better able to cope with oxidative stress may be able to sustain these costs. On the other hand, brood size was negatively associated with tGSH levels in females and positively with oxidative damage in males. Moreover, female body mass also predicted the antioxidant status expressed as tGSH in both the incubation and the nestling stages. These findings indicate

that oxidative status is related to resource allocation (Wiersma *et al.*, 2004) and to conditions dependent on reproductive effort, which in turn is affected by parental metabolic exertion.

We found a correlation between oxidative traits of laying females and their investment in egg attendance. Those females that spent a higher proportion of time incubating suffered a higher oxidative stress, expressed as higher levels of MDA and less intracellular tGSH. Females with higher oxidative damage may decrease the time spent outside the nest box to reduce energetic demands derived from flight, because it has been shown in birds that flight effort may increase oxidative damage and deplete antioxidant status (Costantini *et al.*, 2008, 2012). However, incubation has been shown in this species to be energetically costly (Moreno & Sanz, 1994), so higher nest attendance could also contribute to oxidative stress through metabolic costs of incubation. Nevertheless, Moreno *et al.* (2013*b*) showed that female oxidative damage measured when feeding nestlings was not correlated with incubation attendance in unmanipulated females. This suggests that the effects of incubation attendance on oxidative status may vanish some time after hatching of the young.

Unexpectedly, we found no relationship between provisioning rates and oxidative stress of parents despite the association between provisioning rates and metabolic exertion found in this population (Moreno *et al.*, 2001). This may be due to the fact that under natural conditions (i.e., when breeding effort is not experimentally enhanced), adults adjust their parental investment to maintain redox balance without compromising their welfare (Metcalfe & Monaghan, 2013).

To conclude, the evidence presented here, although observational, strengthens the idea that multiple signalling ornaments in the form of achromatic plumage patches involve costs related to oxidative stress (Morales *et al.*, 2008; Moreno *et al.*, 2011, 2013*a*, 2013*b*) for both male and female Iberian Pied Flycatchers. Moreover, although the importance of reproductive costs in terms of oxidative stress remains controversial (Selman *et al.*, 2012; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014), our study highlights the key role of the specific phase of the breeding period in these links, showing the association between reproductive effort during incubation and female

oxidative status under natural conditions. This supports the critical role of incubation in avian life history evolution (Reid *et al.*, 2002).

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CAPÍTULO VI

ASOCIACIONES SEXO-ESPECÍFICAS ENTRE DINÁMICA TELOMÉRICA Y ESTADO OXIDATIVO EN ADULTOS Y POLLUELOS

López-Arrabé, J., Monaghan, P., Cantarero, A., Boner, W., Pérez-Rodríguez, L. & Moreno, J. Sex-specific associations between telomere dynamics and oxidative status in adult and nestling pied flycatchers. *Physiological & Biochemical Zoology*, submitted

RESUMEN

El estrés oxidativo puede contribuir a una aceleración del acortamiento de telómeros que conduce a la senescencia celular y el envejecimiento. Es conocido que un aumento en la inversión reproductiva acelera la senescencia, lo que generalmente se traduce en un menor potencial reproductivo y supervivencia futuras. Para comprender mejor el papel del estado oxidativo y la dinámica telomérica en la mediación del conflicto entre mantenimiento y reproducción, es importante determinar cómo se relacionan estos factores en individuos parentales y con su descendencia. Investigamos la relación entre el estado oxidativo y las medidas teloméricas en papamoscas cerrojillo (*Ficedula hypoleuca*). Se evaluaron la capacidad antioxidante total (TAS) en el plasma, los niveles totales de glutatión (tGSH) en glóbulos rojos y el daño oxidativo en lípidos plasmáticos (malondialdehído, MDA) en adultos y polluelos. Los telómeros se midieron en células rojas en individuos adultos. Nuestros resultados mostraron diferencias sexuales en las variables oxidativas en adultos, que es probable que sean mediadas a través de esteroides sexuales, con la testosterona y los estrógenos aumentando y reduciendo, respectivamente, la producción de especies reactivas de oxígeno y nitrógeno. Encontramos una asociación negativa entre la longitud de los telómeros (TL) y el MDA medido el año anterior en adultos. Además, la TL se asoció positivamente con el TAS en hembras, mientras que el acortamiento telomérico (ΔTL) se correlacionó positivamente con el MDA en machos en el año en curso. Estas asociaciones podrían estar reflejando diferencias entre los sexos en el comportamiento o la fisiología reproductiva. Encontramos una correlación positiva entre el ΔTL de adultos y el MDA en polluelos. Esto puede interpretarse como un ejemplo de cómo el envejecimiento fisiológico parental podría afectar a la calidad de la descendencia en términos de estrés oxidativo, resaltando las restricciones impuestas por mayores tasas de acortamiento de los telómeros durante la reproducción y crianza.

ABSTRACT

Oxidative stress can contribute to an acceleration of telomere erosion leading to cellular senescence and ageing. Increased investment in reproduction is known to accelerate senescence, generally resulting in reduced future reproductive potential and survival. To better understand the role of oxidative status and telomere dynamics in the conflict between maintenance and reproduction, it is important to determine how these factors are related in parents and their offspring. We investigate the relationship between oxidative status and telomere measurements in pied flycatchers (*Ficedula hypoleuca*). Total antioxidant capacity (TAS) in plasma, total levels of glutathione (GSH) in red blood cells (RBCs) and oxidative damage in plasma lipids (malondialdehyde, MDA), were assessed in both parents and nestlings. Telomeres were measured in RBCs in adults. Our results showed sexual differences in oxidative variables in adults, that are likely to be mediated via sex steroids, with testosterone and estrogens increasing and reducing, respectively, the production of reactive oxygen and nitrogen species. We found a negative association between telomere length (TL) and MDA in adults in the previous season. Moreover, TL was positively associated with TAS in females, while telomere shortening (ΔTL) positively correlated with MDA in males in the current year. These associations could be reflecting differences between sexes in behaviour or reproductive physiology. We found a positive correlation between parental ΔTL and nestling MDA, that is an example of how parental physiological ageing could affect offspring quality in terms of oxidative stress, highlighting the constraints imposed by higher rates of ΔTL during reproduction and rearing.

Keywords: *Ficedula hypoleuca*, oxidative stress, telomere length, telomere shortening

INTRODUCTION

Oxidative stress is defined as the breakdown in the equilibrium between antioxidant defences and the generation of pro-oxidants, which leads to oxidative damage to biomolecules (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2015). Oxidative stress may have important health-related implications (Ames *et al*., 1993), affecting several fitness-related traits and shaping animal life-history evolution (Costantini, 2008; Dowling & Simmons, 2009; Monaghan *et al*., 2009; Costantini *et al*., 2010; Metcalfe & Alonso-Alvarez, 2010). In addition to its effect on macromolecules, oxidative stress could increase senescence rates through its effect on telomere erosion, which ultimately leads to cellular senescence and ageing (Von Zglinicki, 2002; Monaghan & Haussmann, 2006; Aubert & Lansdorp, 2008).

Telomeres occur at the ends of the linear chromosomes of most eukaryotes. They are specialized nucleoprotein structures consisting of highly conserved non-coding sequence. Together with various associated proteins, telomeres cap the chromosome ends (Blackburn, 1991; Armanios & Blackburn, 2012), preventing chromosome degradation and maintaining genome stability during cell division. In addition to this protective role of coding DNA, telomeres are also thought to have a central role in the regulation of chromosome segregation during both mitosis and meiosis (Aubert & Lansdorp, 2008). In the absence of restoration and repair processes, telomere length declines with each cell division; the amount of telomeric DNA lost per round of cell division can be increased by certain factors, most notably oxidative stress (Von Zglinicki, 2002).

Telomeres of a critically short length have been associated with premature ageing syndromes and reduced survival (Bauch *et al*., 2013). It has been previously reported in avian studies that birds show somatic telomere attrition and that has been linked to longevity, as in the case of seychelles warblers, *Acrocephalus sechellensis* (Barrett *et al*., 2013) and zebra finches, *Taeniopygia guttata* (Heidinger *et al*., 2012). However, telomere length has been suggested to reflect an individual's biological state, rather than its chronological age (Bauch *et al*., 2013). Studies in the field and in the laboratory have shown that increased investment in reproduction can accelerate senescence, generally

resulting in reduced future reproductive potential and survival, and increased oxidative stress, and thereby telomere attrition, and may constitute a link between reproductive investment and its fitness consequences (Bauch *et al*., 2013). When reproductive investment exceeds what is sustainable for parents, the costs, in terms of decreased longevity, may become apparent through accelerated ageing (Santos & Nakagawa, 2012; Badás *et al*., 2015). Thus, studies increasing brood size in a range of organisms have shown that costly reproductive events may have a negative impact on telomere dynamics for breeding adults (Reichert *et al*., 2014) and developing offspring (Nettle *et al.,* 2013; Boonekamp *et al.,* 2014; Herborn *et al.,* 2014). To better understand the role of oxidative status and telomere dynamics in mediating the conflict between maintenance and reproduction, one important challenge is to determine how telomere attrition and oxidative stress are related to each other. Further, intergenerational effects of these interlinked processes might influence fitness via effects on offspring health and longevity. In addition, it has been shown that males and females respond differently to reproduction and stressful conditions in terms of oxidative stress and telomere dynamics (Kotrschal *et al.,* 2007; Young *et al.,* 2013; López-Arrabé *et al.,* 2016). Understanding these physiological sex-specific costs is essential to fully appraise these life-history trade-offs.

In this study we investigate the relationship between oxidative status and telomere length in breeding adults of the two sexes in short-lived altricial birds and, using longitudinal data, estimate the rate of telomere attrition. In addition, we explored possible cross-generational associations between telomere measures in adults and offspring oxidative status. For these purposes, we collected data during two reproductive seasons from adults of known age and nestlings of a Pied flycatcher (*Ficedula hypoleuca*) population breeding in central Spain.

In order to evaluate the redox balance of individuals in this study, we obtained measures of both oxidative damage and antioxidant defences. We measured plasma malondialdehyde (MDA) levels, a by-product of lipid peroxidation (Halliwell & Gutteridge, 2007), as a measure of oxidative damage (Mateos *et al*., 2005; Halliwell & Gutteridge, 2007; Sepp *et al*., 2012). To monitor antioxidant defences, we used two independent markers: total antioxidant status of plasma (TAS) and total glutathione (tGSH) levels in red blood cells (RBCs). Telomere length was measured in RBCs. We examined the relationship between telomere dynamics (length and loss) and oxidative status and

telomere attrition. We predicted that those individuals with shorter telomeres or higher rates of telomere shortening (i) would show higher levels of oxidative stress and/or (ii) would produce nestlings with increased levels of oxidative stress (higher levels of oxidative damage and/or reduced antioxidant defences).

METHODS

General field methods

The study was conducted during the springs of 2013 and 2014 in a montane forest of Pyrenean oak (*Quercus pyrenaica*) at 1200 m.a.s.l. in Valsaín, central Spain (40˚ 54' N, 4˚ 01' W) where long-term studies on cavity-nesting birds have been ongoing since 1991. In the study area there are 570 nest-boxes (see appendix in Lambrechts *et al*., 2010 for dimensions, structure and placement of nest-boxes) occupied by pied flycatchers, great tits (*Parus major*), nuthatches (*Sitta europaea*) and blue tits (*Cyanistes caeruleus*).

We followed breeding activities from nest construction to fledging in nest-boxes occupied by pied flycatchers. Egg laying in our population of pied flycatchers typically began in late May. Females laid on average 6 eggs and chicks usually fledged at the age of 17 days. Adult males and females were captured in their nest-boxes with traps while provisioning nestlings of 7-8 days, ringed if necessary or identified and weighed to the nearest 0.01 g with a digital balance. From every adult bird, we took a blood sample of about 120 µl from the brachial vein that was collected in heparinized microcapillaries (N = 62 samples from 31 individuals -19 females and 12 males- captured in 21 nest-boxes in 2013 and re-captured in 25 nest-boxes in 2014). Blood samples were stored in eppendorf tubes in an ice-box until returning to the lab in the same day. Plasma was separated from blood by centrifugation (10 min at 12000 rpm) and then both fractions were stored at –80°C until analysed for assaying MDA, TAS and uric acid from plasma and tGSH and telomere length (TL) in RBCs (see below). Because of total volume of some plasma samples was not sufficient to carry out all physiological or biochemical analyses, we established a priority order of assays as follows: MDA, triglycerides, TAS and uric acid levels. This explains why sample sizes for different measures differ. If haemolysis occurs

during sampling, a possible efflux of intracellular pro-oxidants and antioxidant molecules into plasma could alter levels of oxidative markers measured in blood samples, thereby confounding interpretation of results. Thus, haemolysis levels in plasma samples were noted by a visual detection of red colour of plasma in a continuous gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree in order to minimize inter-observer variability.

In both years, at day 13 (hatching date = day 1), nestlings from studied adults were ringed, weighed, measured and a blood sample was collected following the same protocol as in adults. In 2013 we collected blood from two randomly selected nestlings of each nest (N = 40 nestlings from 21 nest-boxes), whereas in 2014 we took blood samples from all chicks in the nest (N = 74 nestlings from 14 nest-boxes).

Adults ranged from 1 to 5 years old. For most of the adults exact age was known as they were ringed as nestlings in the study area. To estimate the age on first capture of those individuals that were not raised in the study area, we assumed a conservative age of two years on the basis of patterns of age at first reproduction observed in birds of exactly known age (Potti & Montalvo, 1991).

Lipid peroxidation assays

Plasma concentrations of MDA were calculated as described in López-Arrabé *et al*. (2014a). Briefly, a standard curve was prepared for calibration, using a 1,1,3,3 tetraethoxypropane stock solution serially diluted in 40% ethanol. Butylated hydroxytoluene, phosphoric acid and thiobarbituric acid (TBA) solutions were added to each plasma sample and standard. Then, samples were incubated on a dry bath to allow formation of MDA-TBA adducts. After that, pure n-butanol was added to each sample and standard. Tubes were vortexed and centrifuged and the upper phase was collected and transferred into an HPLC vial for analysis. Samples were injected into an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Data were collected using a fluorescence detector (ref. G1321A, Agilent Technologies). Repeatability (following Lessells and Boag 1987), calculated on a set of samples assayed in duplicate was high (r = 0.722, N = 66, $p < 0.001$). Inter-assay CV was 8.08%.

Total antioxidant status (TAS)

TAS measures the capacity of plasma samples to inhibit a redox reaction induced by free radicals (Miller *et al*., 1993; Cohen *et al*., 2007) and is primarily the result of the pooled effect of all extracellular antioxidant compounds of the blood (Costantini, 2011). TAS was analysed as described in López-Arrabé *et al*. (2014a). As standard for the assays we used Trolox (a water-soluble α-tocopherol derivative) and TAS levels are expressed in Trolox-equivalent units. The assays were run on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont, USA). In order to accurately control the reaction time, only one column of the plate was used at a time. To standards and samples were sequentially added metmyoglobin (a mixed of equal volumes of myoglobin (ref. M0630-250MG, Sigma-Aldrich, St. Louis, Missouri, USA) and potassium ferricyanate), ABTS (the chromogen, 2,2′-azinobis-(3- ethylbenzothiazoline-6-sulphonic acid) and H_2O_2, starting the reaction. Kinetic measurements were immediately started, recording absorbance at 660 nm every 5 s. The temperature was maintained at 37ºC during assays. All samples were assayed in duplicate and results showed a high repeatability (r = 0.981, N = 234, $p < 0.001$) and an inter-assay CV of 3.63%.

Intracellular total glutathione (tGSH) level

Glutathione is a tripeptide thiol functioning in the protection of cells against free radicals, being often considered as the most important endogenous antioxidant (Meister, 1991; Wu *et al*., 2004). tGSH levels in RBCs were determined as described in López-Arrabé *et al*. (2014b) and Romero-Haro and Alonso-Alvarez (2015). Briefly, RBC samples were diluted and homogenized in a stock buffer and mixed with trichloroacetic acid. The mixture was vortexed and centrifuged and the supernatant was separated. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc.). To samples (supernatant) we added a mixture of NADPH and DTNB. Afterward, a GSH reductase solution was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH. The assays were performed at 37ºC and only one column of the plate was used at a time in order to control reaction times accurately.

A set of samples assayed in duplicate showed a high repeatability (r = 0.983, N = 106, p < 0.001) and an inter-assay CV of 3.89%.

Measurement of uric acid levels

TAS levels can be affected by the concentration of uric acid, which may confound the interpretation of this oxidative stress biomarker (Cohen *et al*., 2007; Costantini, 2011). Uric acid is the main form of nitrogen excretion in birds and its levels increase quickly after feeding and during faster (Alonso-Alvarez & Ferrer, 2001), but is also a powerful antioxidant frequently positively related to TAS values (Cohen *et al.,* 2007; Hõrak *et al*., 2007; Pérez-Rodríguez *et al*., 2008). Nevertheless, whether uric acid is actively regulated in response to oxidative stress is still unclear (Romero-Haro & Alonso-Alvarez, 2014) because its variability is often explained by amino acid catabolism or inflammation (Sautin & Johnson, 2008). Therefore, we also quantified this plasma metabolite in order to statistically control for its effect in the analyses. Following previous studies in birds (e.g. Pérez-Rodríguez *et al*., 2008; Romero-Haro & Alonso-Alvarez, 2015), plasma levels of uric acid were measured using commercial kits (ref. 11522 from Biosystems, Barcelona, Spain) based on the uricase/peroxidase method (Fossati et al., 1980). Analyses were run in 96-well plates using the same microplate reader mentioned before. 150 µl of the chromogen were added to 5 µl of each plasma sample or the standard (a 6 mg/dl uric acid solution). Plates were incubated for 5 min at 37ºC, subsequently measuring absorbance at 520 nm. A subset of samples assayed in duplicate showed a high repeatability (r = 0.99, p < 0.001, N = 45). Inter-assay CVs were 2.79%.

Telomere analysis

Blood is a good tissue for measuring avian telomere lengths because, in addition to being a highly mitotic motile tissue, erythrocytes are nucleated and thus allow for large amounts of DNA to be obtained from small samples (Barrett *et al*., 2013). Genomic DNA was extracted from RBCs using the MACHEREY-NAGEL Nucleospin® Blood Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) by resuspending 15 µl of red blood cells in 185 µl of PBS and following the manufacturer's protocol for DNA purification from

whole blood. The concentration and purity of DNA samples were assessed using a Nanodrop-8000 Spectrophotometer (ThermoScientific), with all samples having $A_{260/280}$ and $A_{260/230}$ ratios > 1.7 and 1.9 respectively. DNA samples were stored at −20°C. Relative telomere measurements were made using the qPCR methods as described by Criscuolo *et al*. (2009) with some modifications. All PCRs were carried out on a Mx3500 qPCR system (Agilent). Telomere length was measured as the ratio (T/S) of telomere repeat copy number (T) to control, single gene copy number (S), relative to a reference sample. The control, single copy number gene needs to have the same copy number among individuals in the population and within individuals over time. Here, we used 18S as a non-variable copy number gene (Voillemot et al., 2012). Forward and reverse telomere primers were 5′-CGGTTTG TTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ (Tel-1b) and 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′ (Tel-2b) respectively, and forward and reverse 18S primers were 5′-GAGGTGAAATTCTTGGACCGG-3′ and 5′-CGAACCTCCGACTTTCGTTCT-3′ respectively. Telomere primers (Tel1b and Tel2b) were used at a final concentration of 500 nM and 18S primers (forward and reverse) at a final concentration of 50 nM. DNA samples (10 ng) were assayed using the Absolute blue qPCR SYBR green Low Rox master mix (Thermo scientific). The telomere thermal profile was 15 minutes at 95°C, followed by 27 cycles of 15 seconds at 95°C, 30 seconds at 58°C, 30 seconds at 72°C. The 18S thermal profile was 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, 30 seconds, 72°C. Both assays were followed by melt curve analysis of (58–95°C 1°c/5 s ramp). A reference sample was serially diluted (from 40 to 2.5 ng/well) to produce a standard curve for each plate. This was used to calculate plate efficiencies, all of which fell within the acceptable range (mean±ES.: telomere, 106.56%±2.10%; 18S, 99.35%±9.76%) and only samples that fell within the bounds of the standard curve were included. One individual repeatedly fell outside the standard curve and were excluded from the analysis. Each sample was assayed in triplicate and the mean of the three replicates used. The telomere and 18S reactions were carried out on separate plates, and in both reactions the number of PCR cycles (Ct) required for the products to accumulate enough fluorescent signal to cross a threshold was determined. Individuals with relatively short telomeres are characterized by high Ct values, whereas those with relatively long telomeres are characterized by low Ct values (Cawthon, 2002). The average intra-plate variation of the Ct value was 2.05% for the telomere assays and 0.60% for the 18S assays, and the average inter-plate variation

of the ΔCt was 2.24% for the telomere assays and 0.91% for the 18S assays. R^2 values calculated from the reference curves of the qPCR runs were > 0.99 for both the telomere and GADPH assays. The mean coefficient of variation for the T/S ratios was 20%. From these analyses we obtained three measures: initial relative telomere length measured in 2013 ($TL_{initial}$), telomere length in 2014 (TL_{2014}) and telomere shortening (ΔTL, the difference between TL_{2014} and $TL_{initial}$).

Statistical analyses

In order to statistically control for potentially confounding effects, we explored the effect of the degree of haemolysis on the oxidative stress parameters of both nestlings and adults. In all cases haemolysis affected MDA levels (all $p \leq 0.05$). In these cases we controlled MDA levels, entered as independent variable, by the degree of haemolysis in all analyses. Both TAS and tGSH levels were not affected by haemolysis degree (all $p > 0.05$) however, uric acid affects plasma TAS measures (Cohen *et al*., 2007; Pérez-Rodríguez *et al*., 2008), so this marker of antioxidant capacity was always controlled for uric acid levels when entered in the models. Also, we analyzed the relationships of telomere measures and oxidative variables with sex, age and $TL_{initial}$ of adult individuals.

We ran independent models for two telomere measures (TL_{2014} and ΔTL of adult individuals), as dependent variables, and oxidative stress parameters (MDA, TAS and tGSH levels of adults and nestlings) measured in 2013 and 2014 as independent variables. Analyses were run separately for the two years. In all cases, we controlled for laying date (LD), clutch size (CS) and age of adults by including them as covariates. Sex of adults and interaction of sex with oxidative variables were included as fixed factors. The rate of telomere shortening was calculated as the difference in relative telomere lengths between 2013 and 2014. More positive values mean higher rates of telomere shortening. To account for random variation in the starting telomere length among individuals, $TL_{initial}$ was also included as a covariate in all models. To avoid statistical artefacts due to the regression to the mean, the measure of ΔTL was corrected for this phenomenon following the equation suggested by Verhulst *et al*. (2013)

In all cases we used mixed models using PROC MIXED in SAS (version 9.3) and REML (restricted maximum likelihood). Degrees of freedom were estimated using the Satterthwaite approximation, thus avoiding possible pseudoreplication. All dependent variables were normally distributed. Given that some females and males that were paired, nest was included as random factor and individuals were used as a sample unit. To obtain minimal adequate models, we elaborated full saturated models including in each case the factors mentioned above and we sequentially removed the less significant terms following a standard backward stepwise procedure until only significant variables were retained.

RESULTS

There were no significant associations of telomere measures (TL_{2014} and ΔTL) with sex (all $p > 0.10$) and age (all $p > 0.40$) of adult individuals. ΔTL, but not TL_{2014}, was significantly and positively associated with $TL_{initial}$ ($F_{1, 26} = 54.60$; $p < 0.001$ and $F_{1, 26} = 0.03$; $p = 0.864$, respectively). Oxidative stress parameters (MDA, TAS, and tGSH) were not associated either with $TL_{initial}$ (all $p > 0.09$) or age of adults (all $p > 0.19$). In 2014, MDA levels were significantly higher in males than females ($F_{1, 8} = 6.89$; $p = 0.030$; Figure 1a), while TAS values were significantly higher in females than males ($F_{1, 4.5} = 21.91$; $p = 0.007$; Figure 1b). The remaining oxidative stress variables did not differ between sexes (all $p > 0.06$).

TL_{2014} was correlated with adult MDA levels in 2013, independently of sex (Table 1; Figure 2) but was affected by a significant interaction between TAS and sex of adults in 2014 (Table 1; Figure 3). Moreover, TL_{2014} was positively correlated with LD in 2014 ($F_{1, 11} = 13.30$; $p = 0.004$). ΔTL was significantly affected by an interaction between MDA levels and sex of adults in 2014 (Table 1). This association was positive and significant for males ($F_{1, 1} = 819.45$, $p = 0.022$), while ΔTL was not significantly correlated with MDA levels in females ($F_{1, 7} = 0.818$, $p = 0.396$). Moreover, LD and CS in 2014 were positively correlated with ΔTL ($F_{1, 11} = 44.50$; $p < 0.001$ and $F_{1, 11} = 30.74$; $p < 0.001$ respectively).

Table 1. *Summary of mixed models analysing associations of telomere length measured in 2014 (TL_{2014}) and telomere shortening (ΔTL) with oxidative variables of adult individuals in 2013 and 2014 (all statistical tests were controlled for laying date, clutch size, sex and age of adults and initial telomere length. Nest was included as random factor).*

	TL_{2014}	ΔTL
2013		
MDA	$F_{1, 22} = 4.39$; $p = 0.048$*	$F_{1, 18.5} = 3.65$; $p = 0.072$
MDA*Sex	$F_{1, 16} = 0.36$; $p = 0.558$	$F_{1, 16} = .036$; $p = 0.558$
TAS	$F_{1, 14} = 0.54$; $p = 0.476$	$F_{1, 14} = 0.54$; $p = 0.476$
TAS*Sex	$F_{1, 13} = 0.28$; $p = 0.607$	$F_{1, 13} = 0.28$; $p = 0.607$
tGSH	$F_{1, 25} = 3.03$; $p = 0.094$	$F_{1, 22} = 2.62$; $p = 0.119$
tGSH*Sex	$F_{1, 23} = 0.93$; $p = 0.346$	$F_{1, 20} = 0.83$; $p = 0.372$
2014		
MDA	$F_{1, 10} = 0.97$; $p = 0.349$	$F_{1, 11} = 1.08$; $p = 0.322$
MDA*Sex	$F_{2, 13} = 1.12$; $p = 0.356$	$F_{2, 11} = 3.98$; $p = 0.049$*
TAS	$F_{1, 11} = 1.91$; $p = 0.195$	$F_{1, 10} = 2.62$; $p = 0.137$
TAS*Sex	$F_{2, 11} = 4.28$; $p = 0.042$*	$F_{1, 7} = 0.14$; $p = 0.716$
tGSH	$F_{1, 24} = 0.58$; $p = 0.454$	$F_{1, 21} = 0.28$; $p = 0.605$
tGSH*Sex	$F_{1, 18} = 0.00$; $p = 0.956$	$F_{1, 18} = 0.00$; $p = 0.956$

*Significant difference ($\alpha = 0.05$)

Table 2. *Summary of mixed models analysing associations of telomere length (TL_{2014}) measured in 2014 and telomere shortening (ΔTL) of adult individuals with nestling oxidative variables in 2013 and 2014 (all statistical tests were controlled for laying date, clutch size, sex and age of adults and initial telomere length. Nest was included as random factor).*

	TL	ΔTL
Nestlings 2013		
MDA	$F_{1, 23} = 0.75$; $p = 0.394$	$F_{1, 23} = 0.75$; $p = 0.394$
MDA*Sex	$F_{1, 20} = 0.06$; $p = 0.812$	$F_{1, 20} = 0.06$; $p = 0.812$
TAS	$F_{1, 23} = 0.17$; $p = 0.687$	$F_{1, 23} = 0.17$; $p = 0.687$
TAS*Sex	$F_{1, 22} = 0.39$; $p = 0.693$	$F_{1, 22} = 0.16$; $p = 0.693$
tGSH	$F_{1, 18} = 0.02$; $p = 0.894$	$F_{1, 18} = 0.02$; $p = 0.894$
tGSH*Sex	$F_{2, 25} = 1.34$; $p = 0.280$	$F_{2, 22} = 1.82$; $p = 0.186$
Nestlings 2014		
MDA	$F_{1, 16} = 1.38$; $p = 0.257$	$F_{1, 12} = 11.56$; $p = 0.005$*
MDA*Sex	$F_{1, 11} = 2.39$; $p = 0.150$	$F_{1, 12} = 5.92$; $p = 0.031$*
TAS	$F_{1, 15} = 0.10$; $p = 0.757$	$F_{1, 13} = 1.05$; $p = 0.324$
TAS*Sex	$F_{1, 9} = 0.07$; $p = 0.804$	$F_{1, 9} = 0.07$; $p = 0.804$
tGSH	$F_{1, 14} = 0.73$; $p = 0.408$	$F_{1, 12} = 0.93$; $p = 0.353$
tGSH*Sex	$F_{2, 14} = 1.14$; $p = 0.347$	$F_{2, 12} = 1.62$; $p = 0.238$

*Significant difference ($\alpha = 0.05$)

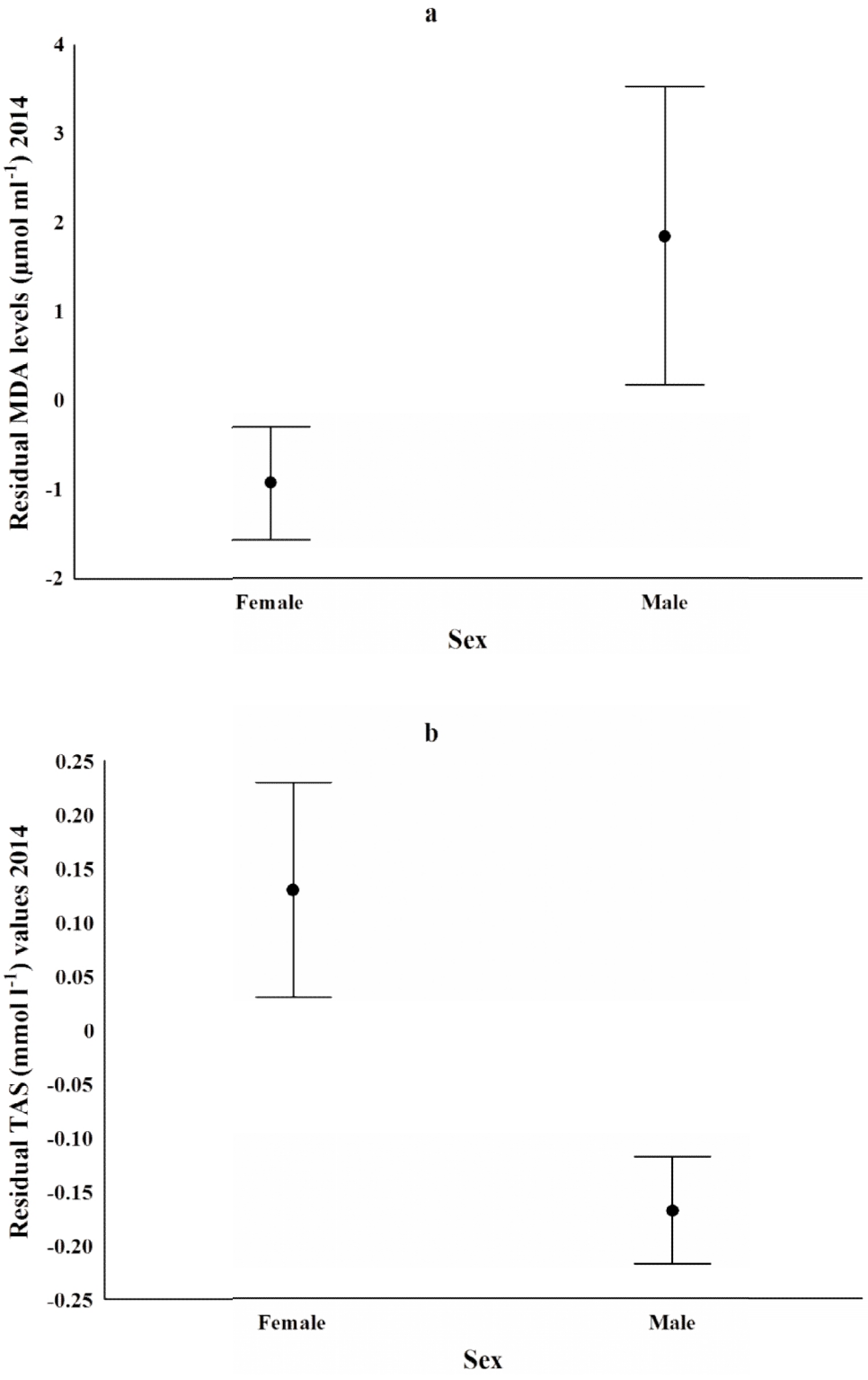

Figure 1. Differences (mean ± SE) between breeding females and males of pied flycatchers in 2014: a) Oxidative damage (malondialdehyde, MDA, residuals after controlling for haemolysis degree); b) Total antioxidant status (TAS, residuals after controlling for uric acid levels).

Maternal and paternal TL_{2014} were not correlated with nestling oxidative stress variables (Table 2). Independently of sex, ΔTL in adults was significantly and positively associated with MDA levels of their nestlings in 2014 (Table 2; Figure 4). We also found here positive associations between ΔTL and LD and CS ($F_{1, 12}$ = 40.85; p < 0.001 and $F_{1, 12}$ = 25.75; p < 0.001, respectively), plus a difference in ΔTL between sexes of adults, being weakly higher in females than males ($F_{1, 12}$ = 5.62; p = 0.035). Moreover, ΔTL was affected by a significant interaction between MDA levels of nestlings in 2014 and sex of adults (Table 2). This association was positive for both sexes, being non-significant for males and females separately (Males: $F_{1, 3}$ = 6.973, p = 0.078; Females: $F_{1, 7}$ = 0.908, p = 0.372).

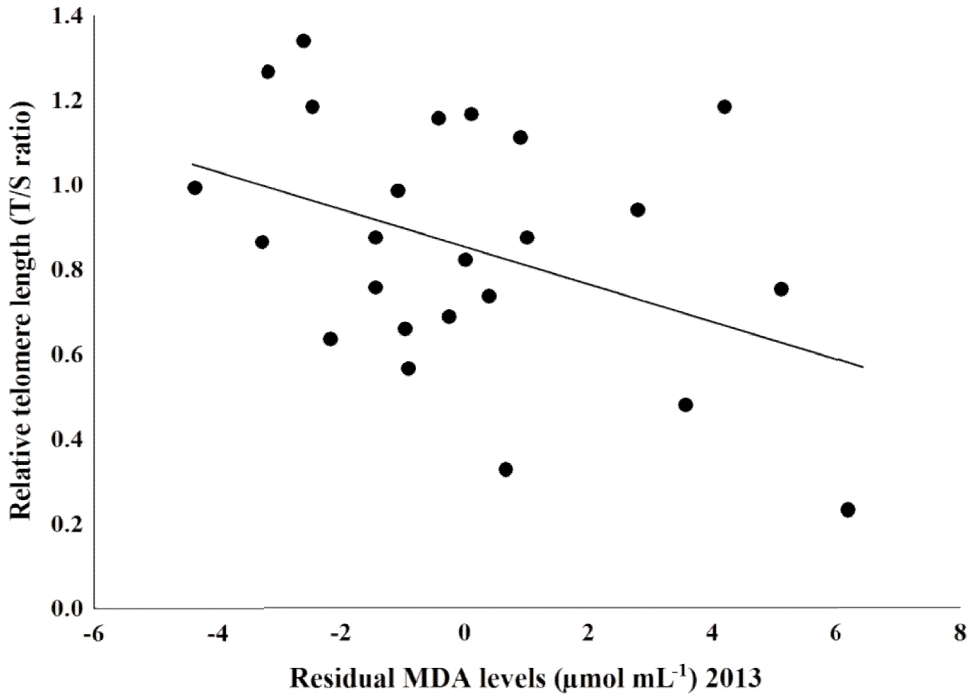

Figure 2. Association between relative telomere length and oxidative damage (malondialdehyde, MDA, residuals after controlling for haemolysis degree) levels of adult pied flycatchers in 2013 ($F_{1, 22}$ = 4.39; p = 0.048).

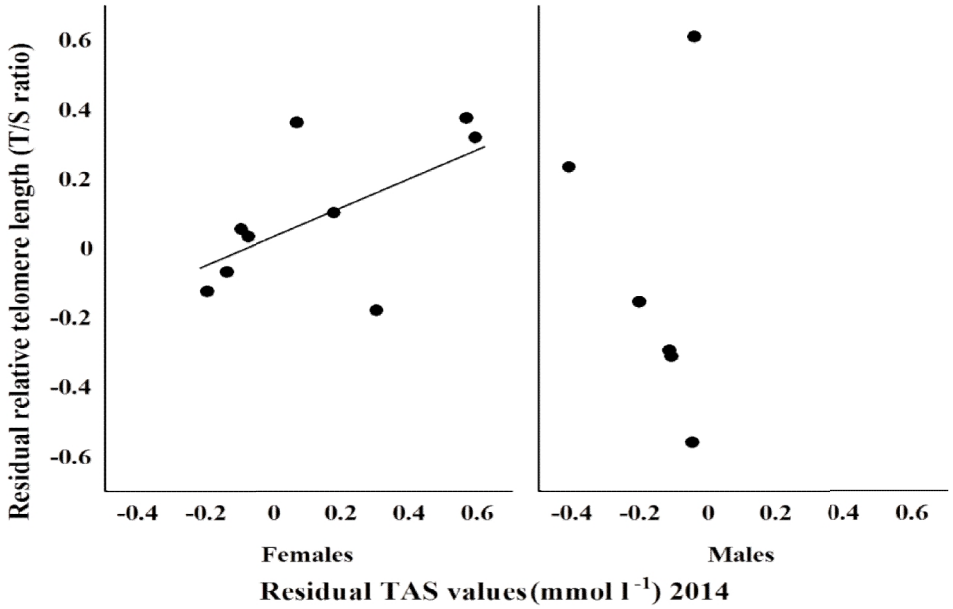

Figure 3. Association between relative telomere length (residuals after controlling for laying date) and total antioxidant status (TAS, residuals after controlling for uric acid levels) of adult female and male pied flycatchers in 2014 (Females: $F_{1, 6}$ = 9.075, p = 0.024; Males: $F_{1, 3}$ = 0.133, p = 0.739).

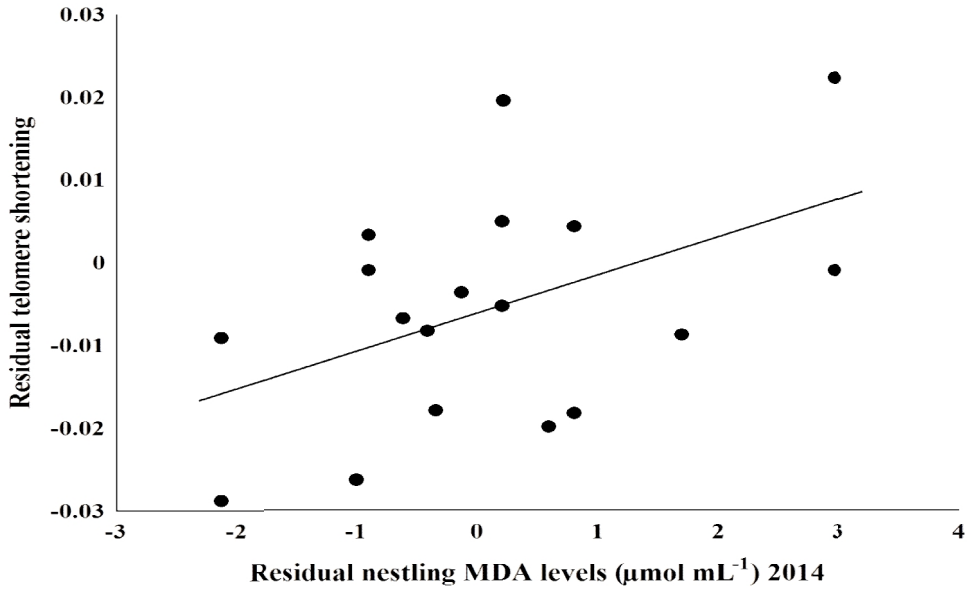

Figure 4. Association between telomere shortening of adult individuals (residuals after controlling for laying date, cultch size and sex of adults) and oxidative damage (malondialdehyde, MDA, residuals after controlling for haemolysis degree) levels of nestlings of pied flycatchers in 2014 ($F_{1, 12}$ = 11.56; p = 0.005).

DISCUSSION

We have conducted an observational study to establish associations between telomere measures in breeding individuals and sex, age and reproductive conditions and oxidative status of parents and nestlings in a Pied flycatcher population. Our results showed sexual differences in levels of oxidative damage and antioxidant capacity in breeding individuals. We found a negative association between telomere length and oxidative damage showed by adult individuals in the previous season. Moreover we found that telomere length was positively associated with antioxidant defences in breeding females, while telomere shortening positively correlated with oxidative damage in males in the present year. Both sexes showed a positive correlation between telomere attrition and nestling MDA levels, though significantly so only for breeding females.

The negative association between telomere length and MDA levels showed in breeding individuals in the last season could imply that oxidative conditions during previous reproductive events may be particularly important in determining telomere length in adulthood. Thus, those individuals with higher levels of oxidative damage would suffer greater rates of telomere loss, modulating their length. Moreover, the positive relationship found between telomere length and TAS values in breeding females in the present year suggests an important protective role of the antioxidant barrier on telomere integrity. Likewise, those males showing higher oxidative damage -i.e. higher MDA levels- in the current season suffered greater telomere attrition rates, which is consistent with oxidative stress being involved in accelerated telomere shortening and ageing. Telomeric DNA is rich in guanine and particularly susceptible to oxidative damage. There is previous evidence that oxidative stress accelerates telomere shortening (Von Zglinicki, 2002; Houben *et al.,* 2008; but see Nettle *et al.,* 2015). Sex differences in these associations could result from the differences in oxidative stress levels shown by female and males. Such difference between sexes in redox status during the breeding season is likely to be mediated via sex steroids, with testosterone and estrogens increasing and reducing, respectively, the production of reactive oxygen and nitrogen species (Gupta & Thapliyal, 1985; Viña *et al.,* 2005) that could in turn affect levels of antioxidant defences and oxidative damage. In addition, males and females experience different life-history trade-

offs likely resulting in variable strategies to deal with redox homeostasis (Costantini, 2008; Metcalfe & Alonso-Alvarez, 2010). Higher antioxidant levels in females may reflect a higher investment in cellular maintenance or, alternatively, an upregulation in response to higher cellular reactive oxygen species during chick rearing (Alonso-Alvarez *et al.,* 2004; Wiersma *et al.,* 2004; Christe *et al.,* 2011; Isaksson, 2013). However, the lower MDA levels found in females seems to support the first scenario, suggesting that they are better protected against oxidative stress than males, the latter showing greater telomere attrition rates at high levels of oxidative damage. Thus, these associations could be reflecting differences between sexes in behaviour, food intake or reproductive physiology, affecting redox balance (Isaksson, 2013) and ageing.

Parents can influence offspring phenotype through genetic and non-genetic effects. In iteroparous animals reproductive performance generally changes with age (Clutton-Brock, 1984), suggesting age-dependent parental effects (Torres *et al.,* 2011). Moreover, the quality and survival of chicks might also be influenced by the deteriorating quality of both male and female germ-line DNA at older ages (Velando *et al.,* 2008). The strong association found between parental telomere shortening and nestling oxidative damage may be interpreted as an example of how parental physiological ageing could affect offspring quality in terms of oxidative stress. Parents showing lower signs of cellular senescence -using telomere shorting as a proxy- could be able to invest more resources in their offspring, for example, by increasing provisioning rates and/or foraging for food items of higher nutritive value to nestlings. This could have a positive effect on offspring oxidative status, reducing their oxidative damage levels during a critical phase like early development. Thus, this association would highlight the constraints imposed by higher rates of telomere shortening during reproduction and rearing. On the other hand, parents and nestlings share rearing and environmental conditions that could affect the association of physiological traits among relatives simultaneously (López-Arrabé *et al.,* 2016).

It is particularly interesting that the association between nestling oxidative stress and parental telomere shortening arose despite the lack of association between telomere measures and the age of adults. This reinforces the idea that it is the degree of somatic senescence, rather than chronological age itself, that affects nestling development. This lack of association between age and telomere measures could indicate that telomere

shortening does not occur at a constant rate with age. In fact, it has been suggested previously that the highest rate of telomere shortening takes place in early life, when somatic growth takes place (Hall *et al.,* 2004; Salomons *et al.,* 2009). Unfortunately, our estimates of telomere shortening were restricted to the adult age, when such period of high growth rate has come to an end. However, we must take into account that mortality could be higher for those individuals with very rapid telomere loss and samples of older birds would therefore be biased towards individuals with slow telomere attrition (Hall *et al.,* 2004).

Sex-specific TL has been documented in many taxa (Barrett & Richardson, 2011; Olsson *et al.,* 2011; Young *et al.,* 2013), particularly in species with large dimorphism in body size, and often related to differences in growth trajectories. In the case of pied flycatchers, sexual dimorphism is mainly based on nuptial plumage, being very subtle in body size (Lundberg & Alatalo, 1992). This could explain the lack of differences in telomere measures between the two sexes in this species.

It has been previously described, for common terns (*Sterna hirundo*), that individuals with shorter telomeres arrive and start breeding earlier in the season and raise larger broods. This association is explained by individual consistency in reproductive performance, implying that consistently successful individuals pay a cost in terms of telomere shortening (Bauch *et al.,* 2013). Here we found that telomere shortening was higher for those individuals with larger clutches and that laid their eggs later in the present year. Larger broods and later broods may be more costly to raise (e.g. Moreno *et al.,* 1998; Alonso-Alvarez *et al.,* 2004; Arnold *et al.,* 2004; Costantini *et al.,* 2014; Reichert *et al.,* 2014, but see López-Arrabé *et al.,* 2016). Moreover, these associations were significant only in 2014 and the lack of a relationship between telomere attrition and the past reproductive performance may indicate that in this case current conditions are more important in telomere dynamics.

In conclusion, we have found associations between telomere dynamics and oxidative status of breeding individuals, showing sex-specific covariation between these traits and some reproductive performance indicators. Moreover, this study presents novel results on the relationship between parental telomere shortening and offspring oxidative damage. Understanding the mechanisms underlying ageing during reproduction and the

links with oxidative stress experienced by breeding birds, and across generations, -as well as the life-history trade-offs associated with the interaction between both factors- is essential to fully understand the importance of oxidative stress and telomere dynamics in shaping individual phenotypes.

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SÍNTESIS GENERAL

En conjunto, los resultados obtenidos a lo largo de esta tesis aportan una visión global acerca del papel del estrés oxidativo como mecanismo implicado en los compromisos a los que se enfrentan los individuos a lo largo de la vida. Por un lado, los Capítulos I, II y III exploran cómo afecta el ambiente de nidificación a las aves que habitan en cavidades. En concreto, el Capítulo I muestra que la presencia de material viejo en el nido no afecta a todas las poblaciones de ectoparásitos por igual, cuestionando la idea general de que la reutilización de nidos está ligada a mayores infestaciones, con consecuencias sobre el éxito reproductor y el crecimiento de polluelos (ej. Mappes *et al*., 1994; Johnson, 1996; Tomás *et al*., 2007; García-Navas *et al*., 2008). En el Capítulo II se muestra cómo el método de manipulación de la carga de ectoparásitos del nido puede tener efectos no controlados sobre las aves, lo que puede llevar a subestimar las consecuencias potenciales de la presencia de dichos ectoparásitos sobre sus hospedadores aviares. El Capítulo III evidencia experimentalmente efectos negativos de la carga de ectoparásitos sobre el estado oxidativo de hembras adultas y polluelos en desarrollo, lo que podría acarrear consecuencias sobre la supervivencia y reproducción futuras (Richner & Tripet, 1999; Fitze *et al*., 2004a, 2004b). En el Capítulo IV se muestran las asociaciones entre los diferentes componentes del estado oxidativo de polluelos en desarrollo y varios factores ambientales e intrínsecos, algo que resulta esencial para entender la importancia del estrés oxidativo en la formación del fenotipo (Lindström, 1999; Metcalfe & Monaghan, 2001; Romero- Haro & Alonso-Alvarez, 2015). Por otro lado, en el Capítulo V se sugiere que diferentes rasgos acromáticos del plumaje pueden señalizar la capacidad individual para hacer frente al estrés oxidativo y se resalta la importancia de las distintas fases del ciclo reproductor para entender el papel del estrés oxidativo como coste y limitación en la reproducción. Por último, el Capítulo VI evidencia cómo el estado oxidativo está involucrado en un acortamiento más rápido de los telómeros (consecuentemente con un mayor envejecimiento celular) de los individuos en edad adulta como coste de la reproducción, además de sugerir que la edad fisiológica de los padres puede afectar a la calidad de la descendencia en términos de estrés oxidativo.

El ambiente de nidificación puede ser determinante para el éxito reproductor y la supervivencia, afectando de forma directa al desarrollo de los polluelos. Los parásitos suponen una importante fuerza selectiva para el desarrollo de mecanismos de defensa, tanto fisiológicos como comportamentales, en aves, especialmente en aquellas que anidan en cavidades y que se ven afectadas por elevadas cargas de ectoparásitos (Atkinson & Van Riper, 1991; Møller, 1997; Hõrak *et al*., 2006; Sepp *et al*., 2012; Cantarero *et al*., 2013a). Para las aves que anidan en cavidades la disponibilidad de oquedades adecuadas para la cría puede ser un factor limitante. Como ya se ha comentado, esta parece ser una de las razones por las que en ocasiones las parejas reproductoras utilizan agujeros para la cría en los que ya hay material viejo (Loye & Carroll, 1998; Tomás *et al*., 2007; pero ver también Loukola *et al.*, 2014), lo que comúnmente se ha relacionado con mayores infestaciones de ectoparásitos hematófagos (Mazgajski, 2007). Sin embargo, en el Capítulo I nuestros resultados muestran que la presencia de material viejo en el nido no afecta de igual forma a todas las poblaciones que componen la comunidad de ectoparásitos en nidos de Papamoscas cerrojillo. Además, se ha encontrado que la reutilización de nidos no parece tener efectos negativos sobre la condición de los polluelos en desarrollo. Ambos resultados están relacionados, ya que los ácaros constituyen uno de los ectoparásitos más virulentos sobre nuestras poblaciones de estudio (ej. Lobato *et al*., 2005, 2008; Moreno *et al*., 2008, 2009; Martínez-De la Puente *et al*., 2009, 2010) y su abundancia no se vio afectada por la presencia o ausencia de material viejo en el nido.

Sin embargo, en aves cuyos nidos contienen varias especies de ectoparásitos a la vez, pueden existir asociaciones entre ellas que hagan necesario considerar a toda la comunidad de ectoparásitos para determinar los efectos adversos sobre sus hospedadores. Se han reportado efectos negativos tanto de ácaros como de pulgas y moscas hematófagas sobre polluelos y adultos, a nivel comportamental, fisiológico y de desarrollo en diferentes poblaciones de aves que crían en nuestras zonas de estudio (Lobato *et al*., 2005; 2008; Moreno *et al*., 2008, 2009; Tomás *et al*., 2007, 2012; Cantarero *et al*., 2013a, 2013b). Además, habitualmente encontramos prevalencias mayores al 50% de los tres tipos de ectoparásitos en los nidos de Papamoscas cerrojillo, por lo que la mayor parte de los nidos presentan dos o más poblaciones de ectoparásitos al mismo tiempo. De este modo, establecer relaciones entre la reutilización de nidos y la

abundancia de una sola población de ectoparásitos puede ser insuficiente para obtener conclusiones fiables sobre sus efectos en la eficacia biológica y el desarrollo de los polluelos.

Por otro lado, la falta de efectos de las distintas especies de ectoparásitos sobre el desarrollo de los polluelos podría explicarse por ajustes a nivel comportamental o fisiológico durante la inversión reproductiva de los adultos, ya sea en un esfuerzo por reducir la abundancia de parásitos en los nidos (Cantarero *et al*., 2013a) o por ejemplo mediante el ajuste del tamaño de nidada en función de dicha carga parasitaria, suponiendo una capacidad de previsión en las hembras adultas (Richner & Heeb, 1995). En cualquier caso, la manipulación de la carga completa de ectoparásitos en los nidos permite controlar los niveles de infestación, eliminando la variación ambiental en el grupo experimental y facilitando la comparación con un grupo control en el que se encuentran niveles naturales de las distintas poblaciones de ectoparásitos, de modo que se puedan determinar los potenciales efectos de toda la comunidad parasitaria sobre los hospedadores. Puesto que la elección de una metodología adecuada para llevar a cabo dicha manipulación puede ser determinante para evaluar de forma exitosa el impacto de los ectoparásitos sobre las aves que se reproducen en cajas-nido, en el Capítulo II se han evaluado dos métodos para eliminar o reducir la carga de ectoparásitos del nido, comparando los efectos del uso de microondas y de un insecticida basado en piretroides sobre la abundancia de parásitos y sobre la condición y la fisiología de las aves. Nuestros resultados destacan los efectos negativos del uso de permetrinas debido a su potencial toxicidad sobre los polluelos en desarrollo ya que se han observado menores pesos tras la eclosión y un menor desarrollo esquelético en polluelos volantones. Asimismo, tanto en polluelos como en hembras adultas se han visto reducidos los niveles de tGSH en aquellas cajas fumigadas con insecticida. Dado el papel del tGSH como antioxidante, ello podría indicar un aumento de su utilización en procesos de detoxificación y protección frente a posibles daños celulares en dichos individuos (Meister, 1991; Wu *et al*., 2004; Ezeji *et al*., 2012). Además, los efectos negativos derivados del uso de insecticidas se asemejan a los observados en aquellas cajas-nido no manipuladas, con elevadas cargas de ectoparásitos, lo que sugiere que su uso en estudios para evaluar el impacto de los parásitos sobre los hospedadores puede enmascarar los efectos de los parásitos,

llevando a conclusiones erróneas por la introducción de una variabilidad sistemática no deseada derivada de su toxicidad.

Así, para analizar el efecto de los ectoparásitos del nido sobre la condición corporal y el estado oxidativo de las aves, en el Capítulo III se ha eliminado la carga completa de ectoparásitos empleando un método físico mediante el uso de microondas. Los resultados principales muestran cómo los ectoparásitos exponen a sus hospedadores aviares a un desafío oxidativo que acarrea consecuencias sobre su sistema antioxidante. Aunque los niveles de MDA no se vieron afectados, tanto los polluelos como las hembras reproductoras mostraron menores niveles de tGSH en aquellos nidos con cargas naturales de parásitos, lo que podría indicar que se ha producido un aumento de ERONs durante la respuesta inflamatoria frente a las picaduras o a consecuencia de un aumento metabólico derivados de la actividad hematófaga de los parásitos (Baron & Weintraub, 1987; Finkel & Holbrook, 2000; Owen *et al*., 2010). Además, las hembras adultas parasitadas también presentaron reducidos valores de TAS, lo que sugiere que éstas están expuestas a una mayor presión posiblemente derivada de la inversión reproductiva, dada la alta demanda de actividad durante esta fase, mostrando por ejemplo un elevado esfuerzo de vuelo durante la búsqueda de alimento que puede acarrear una disminución de las defensas antioxidantes con el fin de mantener la homeostasis redox (Costantini *et al*., 2008). Los parásitos contribuirían a aumentar estos costes según nuestros resultados.

Por otro lado, aunque en el Capítulo II se reportan menores pesos tras la eclosión y un desarrollo esquelético reducido en aquellos polluelos expuestos a ectoparásitos, los resultados del Capítulo III muestran que el mismo experimento no afectó ni a los niveles de triglicéridos ni a los de ácido úrico, lo que sugiere que no hubo diferencia en el estado nutricional entre los individuos de ambos grupos y esto podría explicar la falta de efectos sobre la condición corporal de los volantones.

Además del ambiente inmediato de desarrollo como el que ofrece el nido, existen otros factores, tanto intrínsecos como externos, que pueden determinar los niveles de estrés oxidativo experimentados por los polluelos durante su crecimiento. Así, en el Capítulo IV se profundiza en el estudio de dichos factores moduladores del estado oxidativo durante el desarrollo temprano, analizando las relaciones existentes entre

distintos componentes implicados en el equilibrio redox y factores tales como el sexo, la fecha de crianza y el tamaño de nidada y el estado oxidativo parental. Los principales resultados obtenidos en este estudio muestran que los niveles de tGSH difirieron entre sexos, siendo significativamente mayores en las hembras. Esta diferencia sexual podría estar mediada por los niveles de testosterona, ya que suelen ser mayores en los machos (Naguib *et al*., 2004; Müller *et al*., 2007; Kozlowski & Ricklefs, 2011) y se han asociado en algunos casos con un aumento del estrés oxidativo (ej. Alonso-Alvarez *et al*., 2007, 2009; Mougeot *et al*., 2009).

Se ha encontrado una fuerte correlación entre los niveles de tGSH de los individuos parentales y sus polluelos. Una posible explicación para este resultado es que el estado oxidativo que muestran los individuos parentales puede ser el reflejo del esfuerzo reproductor al que se enfrentan, con subsecuentes efectos sobre la condición oxidativa de sus polluelos. Sin embargo, la alta heredabilidad estimada (de más del 60%) sugiere que los niveles de este antioxidante endógeno estarían expuestos a un importante control genético. Por otro lado, el tGSH de los polluelos se asoció negativamente a los niveles de MDA maternos, reflejando posibles costes y/o limitaciones relacionados con el esfuerzo parental. Según la idea de que una elevada calidad parental permite aumentar los beneficios para la descendencia sin aumentar los costes para los reproductores, aquellas hembras que experimentan menores daños oxidativos podrían hacer frente a una mayor inversión reproductiva, con un beneficio sobre el estado oxidativo de sus polluelos. De forma alternativa, polluelos en peor condición podrían aumentar los comportamientos de solicitación, aumentando sus niveles de estrés oxidativo (Moreno-Rueda *et al*., 2012) y provocando, a su vez, un aumento del esfuerzo parental (Cantarero *et al*., 2013a) que conllevaría una elevada tasa metabólica y un incremento del estrés oxidativo maternos (Nilsson, 2002; Alonso-Alvarez *et al*., 2004; Wiersma *et al*., 2004). Solo un enfoque experimental podría dilucidar entre ambas posibilidades.

Ya en la edad adulta, la expresión de ornamentos sexuales durante la época reproductora puede tener costes derivados de su producción y mantenimiento, de modo que proporcionen una información fiable de calidad individual. En el Capítulo V se establecen relaciones entre las variables oxidativas, las manchas blancas del plumaje, y la inversión reproductiva, mostrando que las defensas antioxidantes en hembras se

asocian con múltiples señales, aunque algunas de estas relaciones varían entre distintas fases del ciclo reproductor. En concreto, hemos hallado que durante la incubación las hembras reproductoras más ornamentadas presentan niveles más bajos de tGSH y TAS, mientras que en el período de alimentación de los polluelos, esta relación es inversa para el tGSH. Al principio de la estación reproductora pueden producirse enfrentamientos competitivos entre hembras por el acceso a los nidales (Moreno, 2015), siendo aquellas más ornamentadas susceptibles de sufrir mayores niveles estrés oxidativo derivado de dichos enfrentamientos (Moreno *et al*., 2013; Morales *et al*., 2014). Durante la fase de desarrollo de los polluelos, las interacciones competitivas entre hembras adultas son raras, de modo que las señales puedes indicar su capacidad para hacer frente al estrés oxidativo. Sin embargo, esta variación en las asociaciones también podría reflejar la capacidad de las hembras para redistribuir recursos a los huevos, siendo aquellas más ornamentadas las que podrían invertir más antioxidantes en los huevos, regulando sus propios recursos para recuperarse posteriormente.

En machos, el tamaño de la banda blanca alar se asoció negativamente con el MDA y positivamente con el tGSH. Se han reportado relaciones similares con la mancha de la frente tanto en otras poblaciones de papamoscas cerrojillo (Moreno *et al*., 2011) como en el Papamoscas collarino (*Ficedula albicollis*) (Markó *et al*., 2011). Esto sugiere que, al igual que en hembras, múltiples ornamentos actúan como un complejo de señalización que proporciona información acerca del estado oxidativo del individuo.

Al analizar las relaciones entre el estado oxidativo y la inversión reproductiva, no encontramos asociaciones con las tasas de cebas. Sin embargo, durante la incubación, las hembras que pasaron más tiempo dentro de la caja incubando los huevos sufrieron mayores niveles de estrés oxidativo. La incubación es una fase energéticamente costosa (Moreno & Sanz, 1994), por lo que puede contribuir a un aumento del estrés oxidativo como resultado de los costes metabólicos asociados.

El estrés oxidativo puede contribuir a una aceleración del envejecimiento y la senescencia celular, aumentando el acortamiento de telómeros. Las tasas de acortamiento telomérico se han asociado a la longevidad en aves (Heidinger *et al*., 2012; Barrett *et al*., 2013), habiéndose sugerido que su longitud refleja la edad biológica de los organismos. Además, se ha observado que la reproducción puede acelerar la

senescencia comprometiendo el potencial reproductor y la supervivencia futuros, constituyendo el estrés oxidativo un nexo de unión entre inversión reproductiva y sus consecuencias sobre la eficacia biológica (Bauch *et al*., 2013). Los resultados obtenidos en el Capítulo VI exploran estas relaciones y los resultados muestran una asociación negativa entre la longitud de los telómeros y el daño oxidativo que experimentaron los individuos durante el evento reproductivo del año anterior. Además, se han encontrado relaciones entre el estado oxidativo y la dinámica telomérica de los individuos durante la misma estación reproductora, de modo que los niveles de antioxidantes en sangre mostraron una asociación positiva con la longitud de los telómeros en las hembras, mientras que en machos, el daño oxidativo se asoció, también de forma positiva con su acortamiento. Estos resultados parecen indicar que los costes asociados a la reproducción, en términos de estrés oxidativo, parecen afectar a la dinámica de telómeros (Von Zglinicki, 2002; Houben *et al*., 2008), de modo que aquellos individuos que experimentan un aumento de sus niveles de daño oxidativo durante un evento reproductor sufrirán mayores tasas de acortamiento, lo que afectará a su longitud. Además, sugieren que los antioxidantes juegan un papel importante en la protección de la integridad telomérica y por tanto frente al envejecimiento.

Por otro lado, de forma similar a los resultados mostrados en el Capítulo IV para los polluelos, los niveles de estrés oxidativo parecen ser mayores en machos que en hembras. Además de la implicación ya mencionada de ciertas hormonas esteroideas, estas diferencias sexuales en individuos adultos también pueden estar indicando diferentes compromisos vitales y estrategias para mantener la homeostasis oxidativa (Costantini, 2008; Metcalfe & Alonso-Alvarez, 2010). Así, diferencias comportamentales, nutricionales o de la fisiología reproductiva pueden modular las asociaciones encontradas entre envejecimiento y reproducción a través de variaciones en el estado oxidativo entre ambos sexos.

Además de los factores que influyen en el estado oxidativo de los polluelos en desarrollo descritos en los Capítulos III y IV, el Capítulo VI muestra cómo la edad biológica de los padres puede afectar al estado de los polluelos, en términos de estrés oxidativo, ya que se ha encontrado una asociación positiva entre las tasas de acortamiento telomérico parental y los niveles de MDA de sus polluelos. Esta relación puede estar influenciada por un deterioro de la calidad del ADN de la línea germinal en individuos

más viejos (Velando *et al*., 2008), pero también puede deberse a que padres menos senescentes serán capaces de invertir más recursos en su descendencia.

A lo largo de esta tesis también se ha visto la importancia de la fecha de crianza sobre la condición de padres e hijos, encontrando diferentes asociaciones entre este factor con el estado oxidativo y la tasa de envejecimiento de los individuos. Así, en el Capítulo IV los niveles de antioxidantes en polluelos están asociados con la fecha de eclosión, de modo que nidadas tardías presentan mayores niveles de tGSH y TAS. Este resultado en cierta medida contraintuitivo (se esperaría una mejor calidad de las nidadas tempranas) puede explicarse por las variaciones estacionales en la disponibilidad de ciertos nutrientes además de limitaciones y costes metabólicos asociados a las condiciones climáticas (más frías al principio de la estación reproductora) en las que se desarrolla la nidada (Biard *et al*., 2005; Dawson *et al*., 2005; Sternalski *et al*., 2010). En el Capítulo V las hembras adultas en fase incubación que criaron antes presentaron una mayor capacidad antioxidante, lo que sugiere que solo aquellas hembras de mayor calidad serán capaces de enfrentarse a los costes derivados de criar pronto en la estación reproductora. También, en el Capítulo VI se ha encontrado que los individuos adultos que criaron más tarde, presentaban una mayor tasa de acortamiento telomérico, de modo que sacar adelante la nidada avanzada la estación reproductora puede ser costoso para los individuos parentales, afectando así a la dinámica telomérica y el envejecimiento (ej. Moreno *et al*., 1998; Arnold *et al*., 2004). Estos últimos resultados confirman los esperados costes de reproducirse tarde, algo observado en numerosas poblaciones de aves de zonas templadas.

Por último, con respecto a los costes derivados de criar mayores tamaños de nidada, varios capítulos de esta tesis arrojan resultados que apoyan la idea del estrés oxidativo como mecanismo implicado en el compromiso entre inversión reproductiva, mantenimiento y desarrollo. En los Capítulos III y V se han encontrado asociaciones negativas entre el tamaño de nidada y los niveles de antioxidantes de las hembras reproductoras. El tamaño de nidada también se asoció de forma positiva con el MDA de los machos en el Capítulo V. Por último, en el Capítulo VI se muestra una relación positiva entre las tasas de acortamiento de telómeros y el número de polluelos. Estos resultados sugieren que sacar adelante mayores nidadas es costoso (ej. Alonso-Alvarez *et al*., 2004; Costantini *et al*., 2014; Reichert *et al*., 2014) y que el estado oxidativo y las tasas de

envejecimiento podrían estar influenciadas por la distribución de recursos (Wiersma *et al*., 2004) y el esfuerzo parental.

En definitiva, esta tesis contribuye a comprobar la fuerte implicación del estado oxidativo en los compromisos que sustentan a las estrategias vitales de las aves. El estado oxidativo responde al parasitismo, a sustancias tóxicas, a los costes de reproducirse y crecer, expresa la senescencia y se asocia a señales de calidad expresadas en el plumaje reproductor. Este entramado de relaciones demuestra que la ecología de los organismos es en parte ecología oxidativa. Futuros estudios sobre estrategias vitales, comportamiento y ecofisiología en poblaciones naturales se beneficiarán de incluir entre los parámetros estudiados aquellos relacionados con el estado oxidativo de los individuos.

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CONCLUSIONES

A la vista de los resultados obtenidos, las principales conclusiones que se extraen de esta tesis son:

1. La reutilización de nidos afecta de forma diferencial a la abundancia de las distintas poblaciones de ectoparásitos presentes en el nido de aves que anidan en cavidades, con lo que no existe un efecto unívoco sobre el desarrollo de los polluelos. Estos resultados cuestionan la generalización de que existen mayores infestaciones en nidos con material viejo en la que se han basado ciertas críticas a la utilización de cajas-nido en estudios con aves.

2. La utilización de insecticidas basados en piretroides en experimentos de manipulación de la carga de ectoparásitos del nido puede tener efectos negativos no controlados sobre la condición corporal y el estado oxidativo de aquellos individuos en íntimo contacto con el material del nido debido a su toxicidad. Esto podría llevar a una subestimación de los efectos reales de los ectoparásitos en aquellos estudios que emplean este tipo de tratamientos.

3. La actividad hematófaga de los ectoparásitos del nido puede suponer un desafío oxidativo para las aves que anidan en cavidades de modo que, tanto hembras reproductoras como sus polluelos han de mantener los niveles de daño oxidativo, a expensas de reducir sus defensas antioxidantes.

4. Dadas las potenciales consecuencias de sus efectos a largo plazo, el estrés oxidativo inducido por parásitos puede conducir a una reducción en las tasas de supervivencia o en la asignación de recursos en futuros eventos reproductivos, pudiendo comprometer su eficacia biológica.

5. Tanto la fecha de crianza como el sexo del individuo explican parte de las variaciones en el estado oxidativo experimentado por los polluelos durante su desarrollo temprano, ya que las defensas antioxidantes son mayores en hembras y en nidadas tardías.

6. Tanto el estado oxidativo como la genética parental pueden ser factores determinantes para los componentes endógenos del sistema antioxidante de los polluelos.

7. Múltiples ornamentos sexuales, como las distintas manchas acromáticas del plumaje, pueden estar asociados al estado oxidativo de los individuos, tanto en hembras como en machos de Papamoscas cerrojillo. Las relaciones entre los niveles de estrés oxidativo, la expresión de señales del plumaje y el esfuerzo parental pueden cambiar en función de la fase del ciclo reproductor.

8. La fase de incubación implica costes en términos de estrés oxidativo para las hembras reproductoras, lo que respalda la importancia del papel que esta fase de la reproducción juega en la trayectoria evolutiva de las aves.

9. Los costes oxidativos ligados a eventos reproductivos pasados y presentes pueden ser particularmente importantes para modular la longitud y las tasas de acortamiento de telómeros durante la edad adulta.

10. Las diferencias sexuales en las asociaciones entre dinámica telomérica y estrés oxidativo en individuos adultos pueden deberse a diferencias entre hembras y machos en el balance redox. Estas diferencias, a su vez, pueden estar mediadas por la actividad de las hormonas esteroideas, o bien pueden deberse a estrategias sexo-específicas (en comportamiento, nutrición o fisiología reproductiva) para mantener la homeostasis oxidativa.

11. La edad fisiológica de los padres, representada por el acortamiento de telómeros, afecta a la calidad de la descendencia en términos de estrés oxidativo, ya que puede influir sobre la capacidad parental para invertir recursos en dicha descendencia.

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ADAPTACIONES FISIOLÓGICAS EN EL PAPAMOSCAS CERROJILLO (*FICEDULA HYPOLEUCA*): ESTRÉS OXIDATIVO, REPRODUCCIÓN Y DESARROLLO

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TESIS DOCTORAL

CAPÍTULO I	Sólo algunas poblaciones de ectoparásitos resultan afectadas por la reutilización de nidos
CAPÍTULO II	El tratamiento experimental con piretroides subestima los efectos de los ectoparásitos sobre las aves que anidan en cavidades debido a su toxicidad
CAPÍTULO III	Los ectoparásitos del nido reducen las defensas antioxidantes en hembras y polluelos
CAPÍTULO IV	El estrés oxidativo durante el desarrollo temprano: asociaciones con el sexo, las condiciones de cría y los rasgos fisiológicos parentales en polluelos
CAPÍTULO V	Ornamentos del plumaje e inversión reproductiva en relación al estado oxidativo
CAPÍTULO VI	Asociaciones sexo-específicas entre dinámica telomérica y estado oxidativo en adultos y polluelos